Potential effects of assisted reproductive technology upon the abundance and localisation of two vital sperm proteins

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Abstract

Title: Potential effects of assisted reproductive technology upon the abundance and localisation of two vital sperm proteins

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Assisted reproductive technology (ART) uses advanced techniques such as *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) to combat human infertility. However, the success rate of ART is poor and can, at least in part, be attributed to detrimental (iatrogenic) damage incurred by gametes and embryos during laboratory treatment or manipulation, thus compromising their functional role and reducing the chances of fertilisation. The sperm plays two fundamental roles upon gamete fusion: (1) to deliver paternal genomic DNA of optimal integrity into the oocyte, and (2) to activate the oocyte to initiate embryogenesis. Protamine and phospholipase C zeta (PLCζ) are two critical sperm proteins fundamentally responsible for facilitating these two key roles, respectively. The essential role of these sperm proteins with regards to male fertility, and fertilisation outcome following ART treatment, has been widely reported. This thesis was predominantly designed to investigate the potential effects of cryopreservation and sperm immobilisation via polyvinylpyrrolidone (PVP) upon the abundance and localisation of protamine and PLCζ in mouse and human sperm, respectively. Deficiency of these proteins could lead to reduced sperm DNA integrity and oocyte activation ability, respectively. An immunofluorescent quantitative assay was first designed and optimised for the determination of protamine 1 (P1) and 2 (P2) levels in sperm. This assay demonstrated that the total levels of P1 and P2, but not the P1:P2 ratio, were significantly reduced (by approximately 50%) in mouse sperm following cryopreservation. This novel assay may represent a useful clinical tool to predict DNA integrity and help select sperm with the best quality DNA. Clinical screening of PLCζ was also carried out in the largest dataset reported to date and confirmed that total levels of PLCζ in human sperm varied significantly between samples ($P \leq 0.05$). Cluster analysis led to the development of a PLCζ scoring system with significant potential as a clinical prognostic and diagnostic assay. Regression models also correlated fertilisation rate and PLCζ content in a total of 30 clinical samples. Collectively, these novel tools show significant promise as predictors of oocyte activation ability. Specific case studies involving vasectomy, oocyte activation deficiency (OAD), and globozoospermia, were identified and shown to be associated with significantly reduced levels of PLCζ ($P \leq 0.05$). In two of these case studies, a single nucleotide polymorphism (SNP) was identified in the PLCζ promoter region, potentially indicating a novel mechanism for PLCζ expression in human sperm. Another case of OAD suggested the apparent deficiency of a crucial interacting factor in the oocyte, emphasising that OAD is not exclusively linked to sperm abnormalities. For the first time, efforts were made to assess whether PLCζ expression was linked to male age; total levels and the proportion of sperm exhibiting PLCζ were found not to differ significantly amongst a total of 46 males. Furthermore, in a pilot experiment, levels of PLCζ were significantly reduced (by 23% to 89%) in PVP-treated sperm from 9 controls and 3 infertile patients, with patient sperm showing higher susceptibility to the effects of PVP compared to controls. However, a more robust experiment featuring sperm from 16 fertile donors, failed to show any significant effect of PVP upon PLCζ. Collectively, data arising from this thesis generated a series of potential clinical tools to quantify protamine and PLCζ in sperm, provides strong evidence that levels of protamine are significantly reduced by cryopreservation, and has provided at least some evidence that PVP may cause detrimental effect upon the level of PLCζ in human sperm. Further work on the effects of vasectomy and the relative functional importance of the SNP detected in the PLCζ promoter are highly warranted. Further investigation and clinical translation of these findings may help to improve the success rate of ART.

Table of contents

List of abbreviations

ACO_2	Aconitate hydratase
ACU	Assisted Conception Unit
$AKAP_3$	A-kinase anchoring protein 3
AOA	Assisted/Artificial Oocyte Activation
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C2 domain	PKC-homology type II
Ca^{2+}	Calcium
CaM/CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CAPZA3	Capping protein (actin filament) muscle Z-line, alpha 3
CASA	Computer assisted sperm analysis
Cdk1	Cyclin-dependent kinase 1
CH_3	Methyl group
CHO cell	Chinese hamster ovarian cell
CMA_3	Chromomycin A3
CPS	Cryoprotective solution
CREM	cAMP response element modulator
CREM-ACT	cAMP response element modulator activator
cRNA	Complimentary ribonucleic acid
CSF	Cytosolic sperm factor
DAPI	4'-6'-diamidino-2-phenylindole
DGW	Density gradient washing
DNA	Deoxyribonucleic acid
DPYI9L2	DPY-19-like 2
DTT	Dithiothreitol
dUTP	Deoxyuridine triphosphate
ER	Endoplasmic reticulum
EST	Expressed sequence tag
FITC	Fluorescein isothicyanate
GFP	Green fluorescent protein
HA	Hyaluronic acid
HCL	Hydrochloric acid
HEK cell	Human embryonic kidney cell
HPLC	High performance liquid chromatography
hrPLCζ	Human recombinant phospholipase C Zeta protein
ICSI	Intracytoplasmic sperm injection
IMSI	Intracytoplasmic morphologically-selected sperm injection
IPP	Inositol polytidylphosphate phosphatase
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
IVF	*In vitro* fertilisation
LC/MS	Liquid chromatography/mass spectrometry
MII	Metaphase II
MAPK	Mitogen-activated protein kinase
MOAT	Mouse oocyte activation test
MPF	Maturation promoting factor
mRNA	Messenger ribonucleic acid
MSOME	Motile sperm organelle morphology examination
NLS	Nuclear localisation signal
NTR	Nuclear transport receptor
NusA	N utilisation protein substance A

OAD	Oocyte activation deficiency
OFU	Oxford Fertility Unit
P1	Protamine 1
P2	Protamine 2
P3	Protamine 3
P4	Protamine 4
PAP	Peroxidase-Antiperoxidase
PAWP	Post-acrsosomal sheath WW binding protein
PCOS	Polycystic ovarian syndrome
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PH domain	Pleckstrin homology
PI	Phosphotidyl inositide
PIP_2	Phosphotidylinositol (4, 5) biphosphate
PI_3P	Phosphotidylinositol 3-phosphate
PLC	Phospholipase C
PLCβ	Phospholipase C beta
PLCδ	Phospholipase C delta
PLCη	Phospholipase C eta
PLCγ	Phospholipase C gamma
PLCε	Phospholipase C epsilon
PLCζ	Phospholipase C zeta
PMSF	Phenylmethylsulfonyl fluoride
POR	Poor ovarian reserve
PRM 1	Protamine 1 gene
PRM 2	Protamine 2 gene
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCD	Sperm chromatin dispersion
SCSA	Sperm chromatin structure assay
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SH	Src homology
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase
SPATA16	Spermatogenesis associated protein 16
TCA	Trichloroacetic acid
TEKT1	Tektin 1
TEMED	Tetramethylethylenediamine
TNP1	Transition protein 1
TNP2	Transition protein 2
TPI1	Triosephosphate isomerase 1
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP, nick end labeling
VIM	Vimentin
YFP	Yellow fluorescent protein

List of figures

List of tables

Chapter 1

Introduction

1.1 Human infertility and assisted reproductive technology

According to the World Health Organization (WHO), the prevalence of human infertility has remained relatively stable over recent years (WHO, 2012). However, fertility disorders continue to affect approximately 25% of couples globally and at least 50 million couples fail to achieve successful pregnancy following 5 years of unprotected sexual intercourse (Mascarenhas *et al.*, 2012; WHO, 2012). In the United Kingdom (UK), as reported by the Human Fertilisation and Embryology Authority (HFEA), infertility is estimated to affect 9 million couples (HFEA, 2012). Infertility is defined as failure to conceive following regular unprotected intercourse (HFEA, 2012) within a period of 12 months (Van Voorhis, 2007), and can be attributed to male, female or idiopathic factors (HFEA, 2012). The most common fertility problems among women are caused by endometriosis, uterine lining defects, cervical polyps, cysts and fallopian tube damage or tubal disease, but can also involve multiple female factors and/or unexplained factors. Other female factors may arise from age factors, ectopic pregnancy, diabetes, epilepsy, thyroid and bowel diseases, or lifestyle factors such as stress, being overweight or underweight, and smoking (Healy *et al.*, 1994; Maheshwari *et al.*, 2008; HFEA, 2014).

Infertility problems among men are frequently associated with abnormal seminal parameters such as complete absence of sperm (azoospermia), low sperm concentration (oligozoospermia), poor motility (asthenozoospermia) or abnormal morphology (teratozoospermia). Another conditions which is common among 0.1% of infertile men is known as globozoospermia (sperm exhibiting round head without acrosome) (Dam *et al.*, 2007a). Other male fertility problems may be attributed to conditions such as, varicocele (abnormal enlargement of scrotal vasculature), cryptorchidism (absence of one or both testes from the scrotum), or problems associated with abnormal gene expression during

spermatogenesis (Kashir *et al.*, 2010). Moreover, several other problems may arise from orchitis (inflamed testes), blocked tubules within the epididymis, drug therapy, radiotherapy, surgery, genetic problems, diabetes, obesity or exposure to environmental pollutants such as chemicals or radiation (Brugh & Lipshultz, 2004; HFEA, 2014). The prevalence of infertility among the human population has led to Assisted Reproductive Technology (ART), a suite of sophisticated laboratory techniques developed specifically to provide the best chance of conception for couples seeking infertility treatment. ART has expanded enormously since the birth of the first *in vitro* fertilisation (IVF) baby, Louise Brown, in 1978 (Steptoe & Edwards, 1978). ART techniques such as IVF and intracytoplasmic sperm injection (ICSI), in which a single structurally-intact sperm is microinjected into an oocyte, have proved very successful in combatting various infertility problems. The ICSI is predominantly used to treat male factor infertility (**Figure 1.1**).

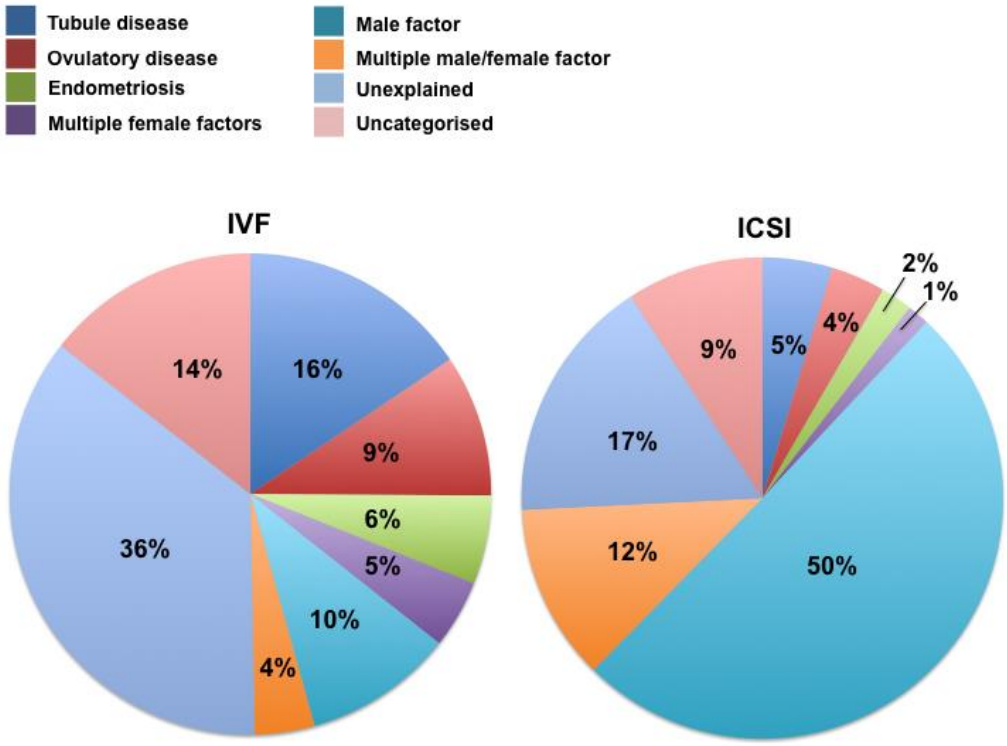


Figure 1.1: Representative images illustrating the proportion of couples seeking *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) treatment based on their specific infertility problem in the UK. Couples with male factor infertility were the largest proportion treated with ICSI. Adapted from HFEA (2012).

Whilst ART techniques have led to the birth of over 5 million babies worldwide (ESHRE, 2013), the success rate of ART remains poor, and rarely exceeds 40% (Nygren *et al.*, 2011). Furthermore, pregnancy outcomes and delivery rates following routine IVF and ICSI falls between a narrow range of only 22.4% and 23.3% respectively (Nygren *et al.*, 2011). In the UK alone, the number of ART cycles has increased rapidly from 1990 to 2011. However, the number of live birth rates is relatively poor (**Figure 1.2**). Consequently there is significant interest in elucidating the factors underlying the inefficiency of ART. The relatively poor success of ART may be attributed to a wide variety of factors including maternal age, chromosomal abnormalities, sperm aneuploidy and/or DNA fragmentation, embryo implantation failure, or problems associated with laboratory or culture environment (Martin *et al.*, 2011; Fiorentino *et al.*, 2014; Chatziparasidou *et al.*, 2014; Lewis, 2014; Yelumalai *et al.*, 2012).

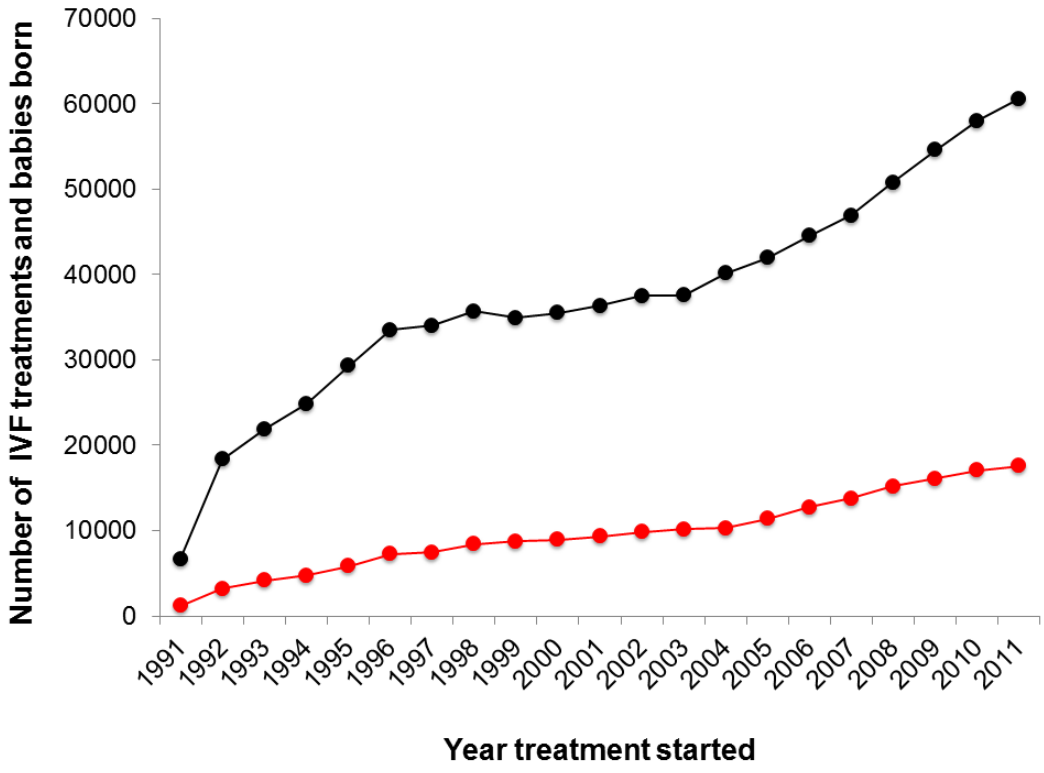


Figure 1.2: The number of assisted reproductive technology (ART) cycles and subsequent live birth rates from 1990-2011 in the UK. Although there was a steady increase in live birth rates, there was a much more dramatic rise in the number of IVF treatment carried out each year. Black line represents number of IVF treatments whereas red line represents number of babies born. Adapted from HFEA (2012).

While, these factors have been clearly linked with poor ART outcome, another potential factor to consider is the effect of ART laboratory procedures (Kashir *et al.*, 2011a; Yelumalai *et al.*, 2012) upon the quality, integrity or function of key gametes proteins. In order to improve the success rate of ART, it is critical to develop new laboratory techniques and/or improve existing ART protocols in order to provide patients with the best clinical care and the best chance of pregnancy. This thesis investigates the role played by two key sperm proteins (phospholipase C zeta and protamine) at fertilisation and attempts to investigate how common ART laboratory techniques, such as cryopreservation or the use of polyvinylpyrrolidone (PVP) might affect their integrity and function of mouse and human sperm.

1.2 The male reproductive system

In order to understand the role of the two sperm proteins under examination, it is first necessary to introduce the way in which sperm develop and mature.

1.2.1 Testicular development and spermatogenesis

Mammalian spermatogenesis is the sophisticated process of male germ cell development, which involves proliferation of diploid spermatogonia cells into mature haploid sperm cells (Krawetz *et al.*, 2009). This complex process occurs in the seminiferous tubules and interstitial tissue of the testis (**Figure 1.3A**) (Cummings, 2001). The seminiferous tubules consist of seminiferous epithelium comprised of germs cells and Sertoli cells adhered on the basement membrane, and surrounded by peritubular cells (Krawetz, 2005). Leydig cells localised in the interstitial tissue between seminiferous tubules, produce androgens to regulate spermatogenesis and later support the development of the surrounding Sertoli cells, and the germ cells (**Figure 1.3B**) (Smith & Walker, 2014).

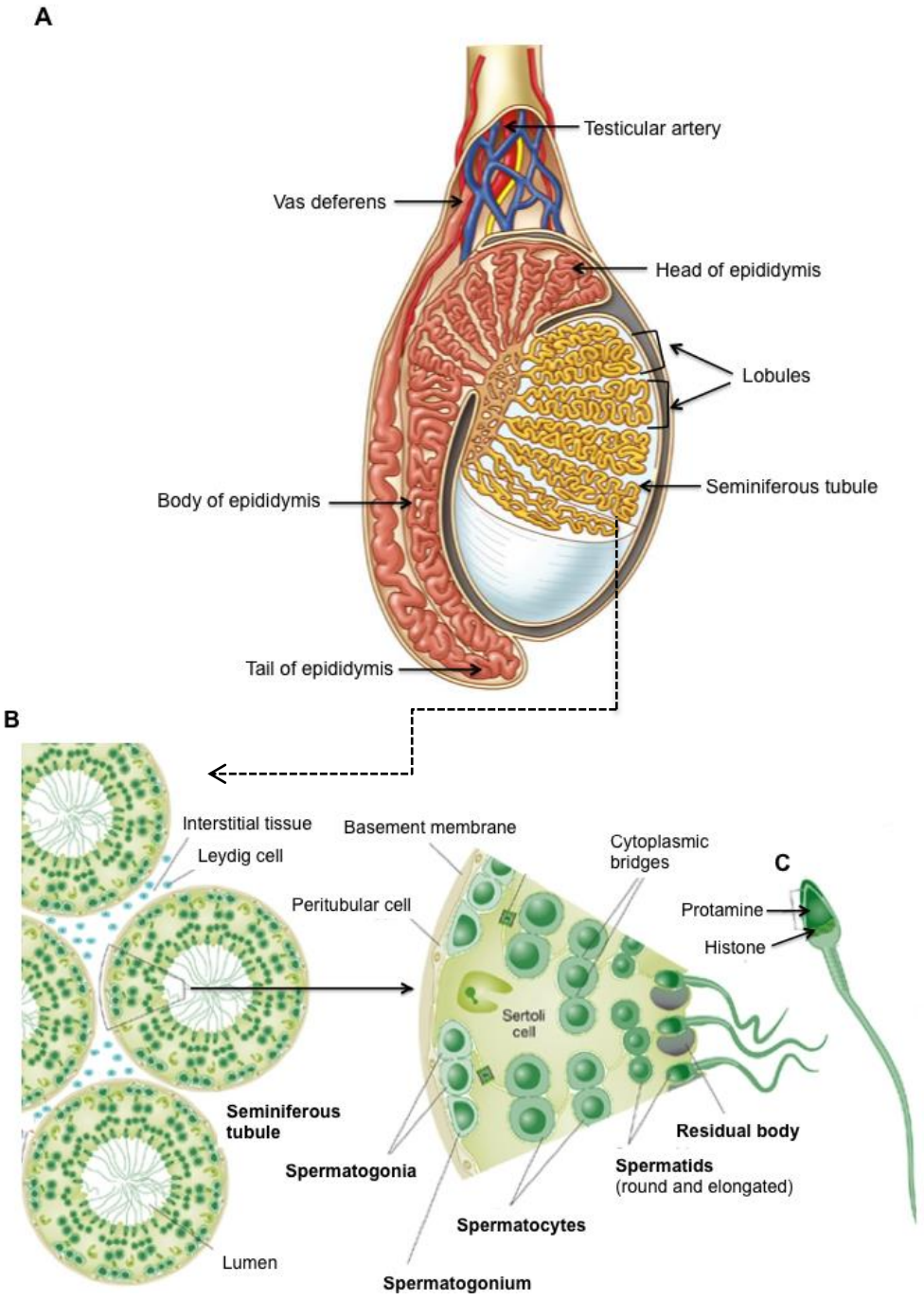


Figure 1.3: Testicular structure, spermatogenesis process and organisation of the sperm genome. (**A**) Testicular and epididymal structure showing the major parts of the testis. Sperm cell proliferation and differentiation occurs in the seminiferous tubules located within the testis. (**B**) Cross section of seminiferous tubule illustrating basement membrane and layer of peritubular cells surrounding each tubule. The epithelium is comprised of progressing germ cells and supporting Sertoli cells. The schematic image further illustrates spermatogonia cell differentiation and development from the basement membrane followed by meiotic division of diploid spermatocyte cells to produce haploid spermatid cells. (**C**) During spermiogenesis, elongating spermatids undergo epigenetic modifications, histone / protamine displacement and restructuring of the sperm nucleus. Nucleosomal histones are mostly replaced by protamine leading to compaction of the paternal genome. Adapted and modified from Krawetz (2005) and Meehan (2001).

The fundamental process of spermatogenesis facilitates the transmission of the paternal genomic DNA and the propagation of species. Mammalian spermatogenesis is divided into three major stages (Rolland *et al.*, 2008). During the initial stages, spermatogonia cells undergo two consecutive mitotic divisions to form primary spermatocyte cells (**Figure 1.4A**). In the second stage, spermatocytes further proliferate through repeated meiotic division developing into haploid round spermatid cells (**Figure 1.4A**). Finally, during a post-meiotic maturation stage termed as spermiogenesis, the round spermatids finally elongate and develop into mature sperm (**Figure 1.4B**) (Hess & Renato de Franca, 2008).

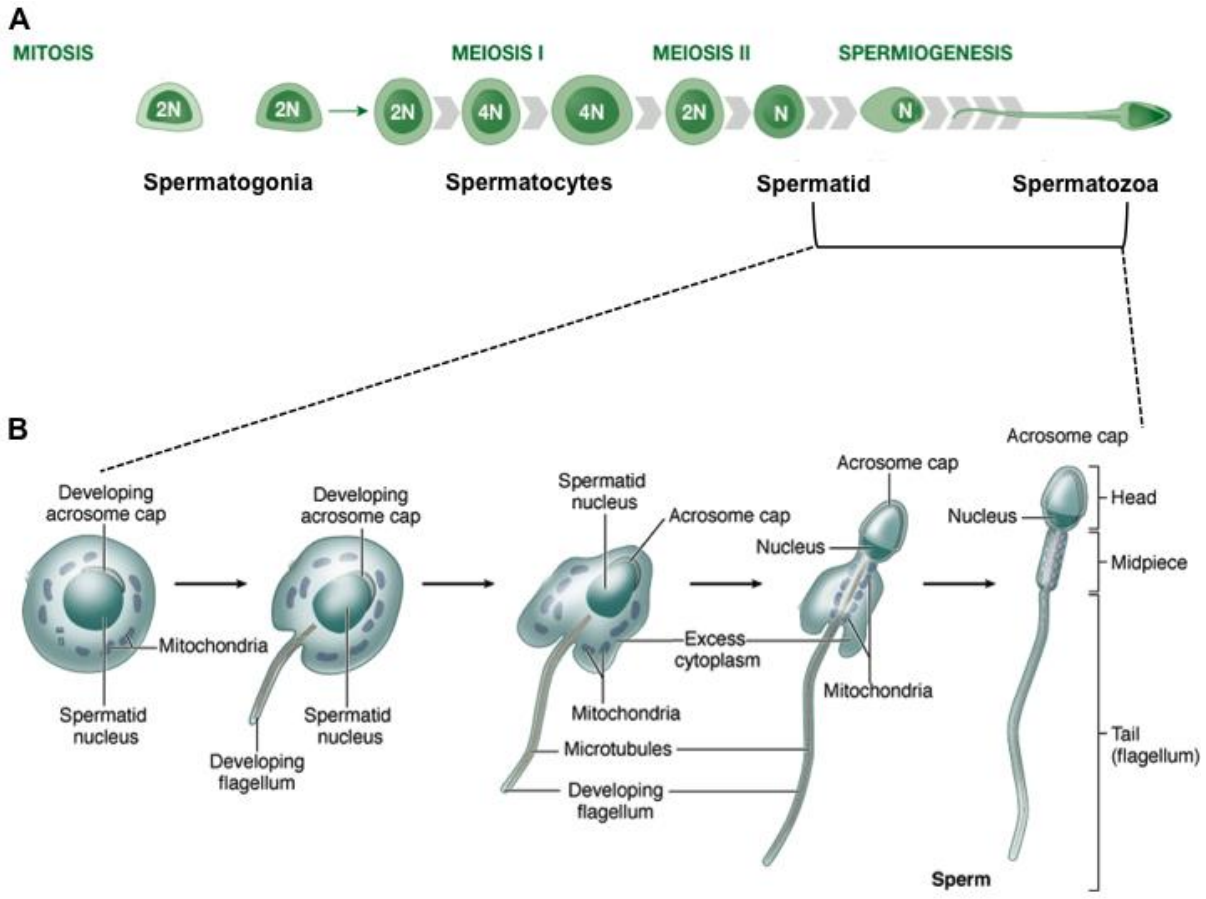


Figure 1.4: Spermatogenesis and sperm development. (**A**) Spermatogenesis process indicating mitotic and meiotic division of diploid spermatogonia to haploid spermatids. (**B**) Development of round spermatids to elongating sperm during spermiogenesis which involves formation and development of the acrosome and flagellum, chromatin condensation, change of nucleus shape, and removal of excess cytoplasm. Adapted and modified from Krawetz (2005) and Mescher (2010).

1.2.2 Epididymal maturation of sperm

In mice, each spermatogenic cycle takes around 35 days to complete with approximately 11 days for the mitotic process, 10 days for the meiotic division, and the final 14 days for post-meiotic division. In humans, spermatogenesis takes approximately 74 days to complete (Clermont & Trott, 1969). Following the final stages of spermatogenesis, the elongated spermatids are released into the lumen of the seminiferous tubule to progress to the epididymis (Cooper *et al.*, 1998).

Sperm first enter the caput epididymis and are generally immature, do not exhibit optimal motility or fertilisation ability and are unable to recognize the zona pellucida. Sperm undergo functional, biochemical and morphological modifications during epididymal transit, a process known as 'epididymal maturation'. During this process, sperm attains several functional properties including the ability to recognize the zona pellucida to facilitate zona binding following gamete fusion, and the acquisition of progressive motility (Cooper *et al.*, 1998; Tulsiani, 2006). Sperm undergo further modification in the epididymis through the influence of biochemical secretions from epithelial cells lining the male reproductive tract. It normally takes human sperm 2–3 days to pass through the epididymis and undergo all structural and functional modifications. Mature sperm are stored in the cauda epididymis to await ejaculation (Toshimori, 1998).

1.2.3 Sperm structure

A differentiated mature sperm adopts a well-characterized structure featuring two major segments; a head and a tail (**Figure 1.5**) (Fawcett, 1975). The shape and size of the sperm head varies across different mammalian species, however in most species, head shape is generally classified as either sickle-shaped as seen in rodents (**Figure 1.5A**), or paddle-shaped as seen in humans (**Figure 1.5B**) (Toshimori, 2009).

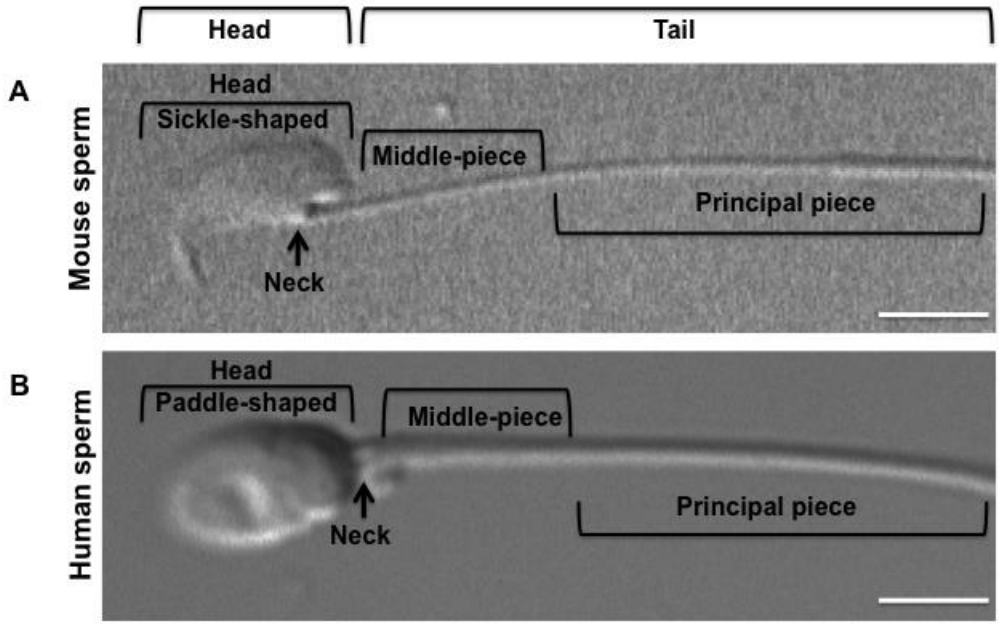


Figure 1.5: Differential interference contrast (DIC) confocal microscopic images showing two major segments of mammalian sperm, the head and tail region. (**A**) Sickle-shaped head of mouse sperm. (**B**) Paddle-shaped head of human sperm. Images captured at x100 magnification. White scale bar = 2.5µm

The sperm head alone is categorized into three segments comprising of nucleus, acrosome, and a small volume of cytoplasm in the form of cytoplasmic layers (**Figure 1.6**) (Toshimori, 2009). The nucleus, containing paternal genomic DNA, is a unique highly organised structure formed by the compaction of DNA by the sperm nuclear proteins protamines 1 (P1) and protamine 2 (P2) to form a hydrodynamic structure (**Figure 1.3C**). Formation of this structure is fundamental for sperm to achieve optimal progressive motility and fertilisation ability (Brewer *et al.*, 2002; Dadoune, 2003). The structural and functional

roles of protamine in mammalian sperm, and its essential association with male infertility are discussed later in this chapter.

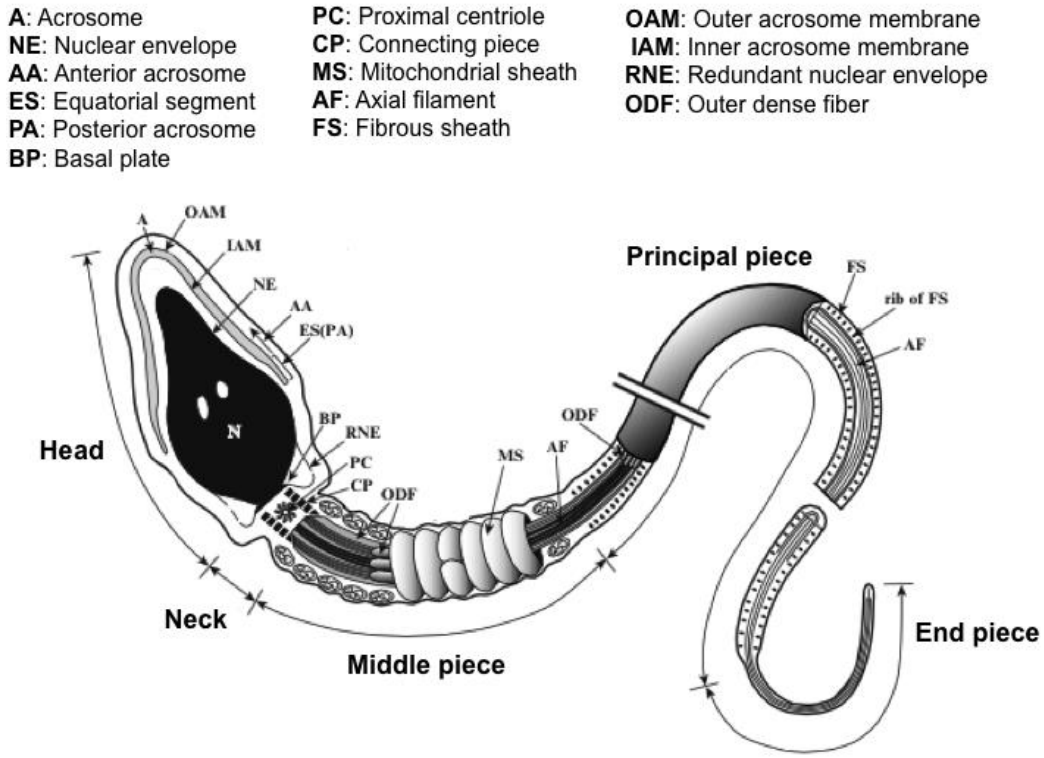


Figure 1.6: Schematic image showing general sperm structure and specific structural aspects distributed across the head, middle piece and principal piece. Adapted and modified from Toshimori (2009).

The acrosome is a cap-like structure that covers the proximal region of the head and is completely enclosed by an acrosomal membrane (OAM: outer acrosomal membrane and IAM: inner acrosomal membrane), which contains hydrolysing enzymes and matrix proteins (**Figure 1.6**). The acrosome plays a vital role during the acrosome reaction at gamete fusion (Yoshinaga & Toshimori, 2003) and will be discussed separately in **Section 1.2.4**. The cytoplasmic region is a thin narrow layer between the nucleus and overlying plasma membrane (**Figure 1.6**). The tail is structurally divided into four main domains; the neck, middle-piece, principal piece, and end piece. The tail contains motility-related apparatus and cytoskeletal structures such as outer dense fibres (ODFs) and fibrous sheaths (FSs) (**Figure 1.6**) (Toshimori, 2009).

1.2.4 Capacitation, acrosome reaction and fertilisation

Sperm undergo fundamental physiological changes (‘capacitation’) in the female reproductive tract following ejaculation to attain the ability to penetrate oocytes. Capacitated sperm usually exhibit several physiological changes such as vigorous tail movement or sperm hyperactivation, which are essential for sperm–oocyte interaction (Cohen-Dayag & Eisenbach, 1994; Suarez, 2008). Capacitated sperm are able to penetrate the oocyte cumulus matrix in order to bind to the zona pellucida (ZP) (Primakoff & Myles, 2002).

The acrosome reaction is a process occurring during gamete fusion when sperm releases hyaluronidase and acrosin from the acrosome to soften and digest the zona pellucida in order to facilitate sperm penetration into the ooplasm (Toshimori, 2011). The acrosome reaction involves hydrolysis or modification of the zona pellucida components and modification of pre-existing molecules on the sperm plasma membrane. These are essential for fusion between the equatorial and post-acrosomal segments of the sperm with the oolemma (Gundersen & Shapiro, 1984; Stein *et al.*, 2004). Subsequently, only acrosome-reacted sperm are capable of penetrating the zona pellucida and fusing with the oolemma. The biological mechanisms taking place at gamete fusion are fundamentality regulated by calcium (Ca^{2+}) signalling including capacitation, chemotaxis and the acrosome reaction (Breitbart, 2002; Costello *et al.*, 2009). Furthermore, following gamete fusion, the oocyte undergoes activation in response to intracellular Ca^{2+} release (Amdani *et al.*, 2013; Kashir *et al.*, 2013a).

1.3 Protamine, a sperm nuclear protein responsible for DNA integrity

Routine semen analysis is considered sufficient to predict the best quality sperm in clinics. However sperm quality is highly dependent upon its specialised structure and DNA integrity, in order to establish successful fertilisation without compromising the paternal genomic DNA upon delivery into the oocyte. In this section, I discuss the fundamental role of protamine, the sperm nuclear protein, in maintaining and protecting the paternal DNA, and also discuss links between protamine and male infertility.

1.3.1 Structure, sub-cellular localisation and function of protamine

Mammalian sperm are unique and specialised cells, predominantly composed of a condensed nucleus, a very small cytoplasmic space, and a long flagellum (Castillo *et al.*, 2014). This unique structure creates a very hydrodynamic structure that enables the sperm to swim faster and thus increase the chance of fertilisation. The formation of a highly condensed nucleus is therefore vital in establishing fertilisation potential (Oliva, 2006; Schagdarsurengin *et al.*, 2012) (**Figure 1.7**). The sperm nuclear chromatin structure is predominantly packed by protamine, a protein composed of P1 and P2 sub-types, with at least 8% of DNA remaining bound to histones (Hammoud *et al.*, 2009; Castillo *et al.*, 2014). Protamines are arginine-rich sperm-specific proteins (Balhorn, 2007) that were first discovered by Friedrich Miescher in 1874 (Dahm, 2005). The fundamental role of the sperm is to deliver paternal genomic DNA into the oocyte and to ensure that the transmission of genetic material is protected from disassociation by endonucleases, exonucleases and/or mutagenic activities (Oliva, 2006; Castillo *et al.*, 2014). Thus, the compaction of DNA in sperm by protamines is a critical process which ensures that these specialised cells can fertilise without genetic risk.

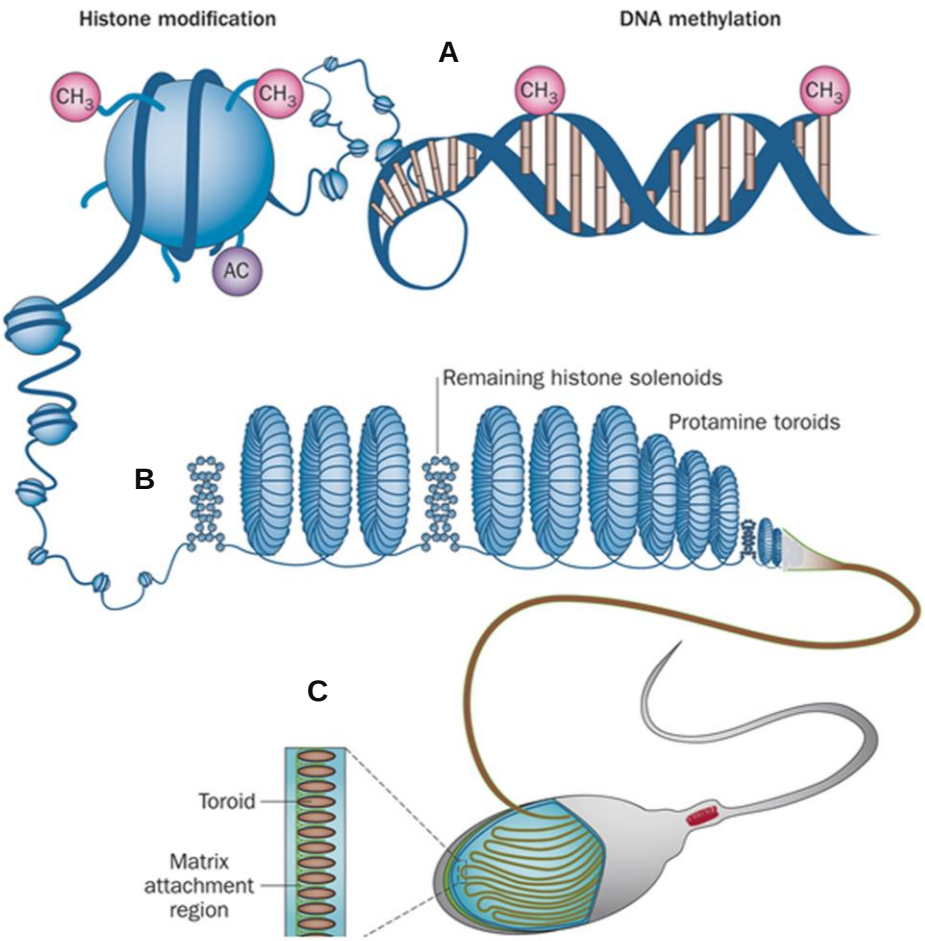
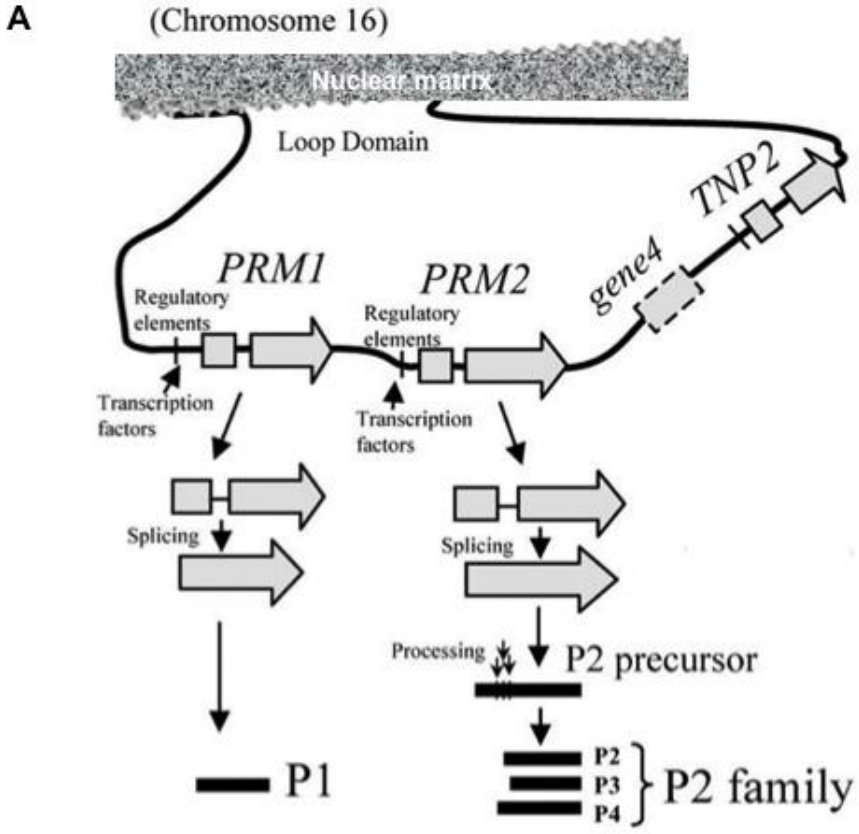


Figure 1.7: Chromatin structural organization in sperm following nucleohistone/nucleoprotamine transition. **(A)** DNA methylation and histone modification. **(B)** Rapid histone to protamine replacement. **(C)** Hydrodynamic structure of sperm head exhibiting highly condensed chromatin with reduced cytoplasmic composition packed into toroidal protamine-DNA structure. Adapted from Schagdarsurengin *et al.* (2012). CH_3 = Methyl group, AC = Acetyl group.

Vertebrates possess 1-15 genes per haploid genome that encodes for protamine. However in most mammals, only one single copy of a gene encodes for P1 and P2 (Balhorn, 2007). While P1 is present in most vertebrates, the P2 family, consisting of P2, protamine 3 (P3) and protamine 4 (P4), are found only in certain mammals, including humans and mice (Carrell *et al.*, 2007), thus indicating the conserved role of P1 (Corzett et al., 2002; Oliva, 2006; Balhorn, 2007). Protamines are characterised by the highest evolutionary variation among species, in terms of the total number of cysteine residues and amino acids present in the sperm nuclei (Oliva, 2006). Such evolutionary diversity contributes to clear differential ratios of P1 and P2 across different mammalian groups (Corzett *et al.*, 2002; Gosalvez *et al.*,

2011). In human, mouse, rat and bull sperm, P1 and P2 genes are arranged in a loop structure on chromosome 16 which harbours the gene for transition protein 2 (TNP2) (Wykes & Krawetz, 2003), and an open reading frame known as 'gene 4' or P3 (Balhorn, 2007) (**Figure 1.8A**). Although the amino acid sequence of P3 is very similar to that of P2, it is unlikely to be involved in DNA packaging since it is composed of glutamic and aspartic residues, which are negatively charged (Balhorn, 2007). The structural domain of P1 and P2 proteins is composed of arginine-rich residues in the central domain and flanked with two short peptides. These short peptides contain cysteine residues and phosphorylation sites (Balhorn, 2007). In humans, approximately, 48% of protamine structure is constructed with arginine (Oliva, 2006). Since arginine is a positively-charged amino acid, it acts as a DNA anchoring domain which binds to the highly negatively-charged chromatin-DNA for condensation (Balhorn, 2007). In addition, the guanine bases in arginine residues forms electrostatic and hydrogen bonds with phosphate groups in the chromatin-DNA strands in order to facilitate complex binding (Balhorn, 2007). P1 and P2 are synthesized by soluble polyribosomes in the cytoplasm of elongating spermatids. While P1 is synthesized as a mature protein, the P2 family arises from proteolysis of precursor proteins (Oliva, 2006) which differ by 1-4 amino acid residues at the N-terminal (Gusse *et al.*, 1986; Oliva, 2006) (**Figure 1.8B**).



B

Protamine 1 (P1)

ARYRCCSQSRSRYYRQRQRSRRRRRRSCQTRRRAMRCCRPRYRPRCRRH

Protamine 2 family

P2: RTHGQSHYRRHCSRRRLHRIHRRQHRSCRRRKRRSCRHRRRHRRGCRTRKRTCRH

P3: GQSHYRRRHCSRRRLHRIHRRQHRSCRRKRRSCRHRRRHRRGCRTRKRTCRRH

P4: ERTHGQAHYRRHCSRRRLHRIHRRQHRSCRRRKRRSCRHRRRHRRGCRTRKRTCRRH

Figure 1.8: Transcriptional activity of protamine genes and the subsequent translational and processing of human protamine. **(A)** Schematic image of *PRM1* and *PRM2* gene organization, and the transcription, translation and synthesis of mature protamine. P1 is synthesized and processed as a mature protein while P2 family arises from processing of P2 precursor. *TNP2* gene encodes for transition protein 2. The *gene*4, (known as P3) may associate with DNA during protamine-DNA packaging. **(B)** Amino acid sequence of human P1 and P2 family. P2 is the most abundant compared to P3 and P4, differing by 1-4 amino acid residues at the N-terminal. The arginine (R) residues are shown in blue font. The cysteine residues are underlined. Adapted and modified from Oliva (2006).

P1 and P2 are immediately phosphorylated following synthesis at their respective phosphorylation site, which is composed of a mixture of serine and threonine residues (Balhorn, 2007). Studies have postulated that protamine phosphorylation is required to prevent the interaction/interruption of serine and threonine sites within chromatin-DNA during the nucleoprotamine-DNA binding process (Oliva, 2006). Finally, following the completion of nucleoprotamine transition, thiols residing in cysteine residues are oxidized to form disulfide bridges in order to strengthen the nucleoprotamine-DNA complex structure by anchoring neighbouring protamine molecules together to prevent the disassociation of DNA from protamine (Balhorn, 2007). Unlike, P1, P2 binds to zinc atoms, that are associated with cysteine thiols in order to facilitate the formation of disulphide bridges (Balhorn, 2007).

P1 and P2 are involved in a sequential process of nuclear chromatin condensation during the elongating spermatid stage (spermiogenesis) (Oliva, 2006; Carrell *et al.*, 2007) (**Figure 1.9**). During spermatogenesis, the P1 (*PRM1*) and P2 (*PRM2*) genes required for the post-maturation stage (spermiogenesis) are actively transcribed and stored in round spermatids or spermatocytes. During early meiosis, the histones are first hyper-acetylated and then modified. Rapid histone hyper-acetylation allows modifications of the acetyl group, which exposes the binding sites in chromatin for subsequent binding of chromosomal proteins. Next, the nucleosome disassembles, followed by unwinding of the chromatin-DNA double helix strand by topoisomerase enzyme II (Oliva, 2006). At the beginning of the elongating spermatid stage, the histones are actively displaced by transition protein 1 (TNP1) and TNP2, followed by the initiation of P1 and P2 binding to the DNA strand (Oliva, 2006). The phosphorylated protamine binding occurs rapidly at this stage, including the formation of disulfide bond cross-linkage during epididymal maturation (Oliva, 2006). Towards the end of spermiogenesis, the majority of histones in sperm nuclei have been replaced with protamine and the condensed nucleoprotamine complex has formed into a toroidal structure

(Oliva, 2006). During fertilisation, the disulfide bridges are reduced and the condensed sperm chromatin structure disassociates readily. Protamines can then be actively removed allowing unpacking of the toroidal DNA structure (Oliva, 2006; Balhorn, 2007). Finally formation of the nucleosome and reorganisation of chromatin structure takes place, leading to the establishment and maintenance of male epigenetic information (Oliva, 2006).

Besides the formation of a complex hydrodynamic structure and the provision of protection for the paternal genomic DNA, protamines also exert several other essential functions, such as a checkpoint for spermiogenesis, fertilising an oocyte and more recently, the inheritance of epigenetic information and imprinted genes (Oliva, 2006). For example, in human sperm, approximately 8%-15% of histones remain bound to the chromatin upon completion of nucleohistone/protamine packaging. Histone modifications and hyper-acetylation plays a crucial role from the start and sets the imprinting program. Potential errors occurring during these process can lead to higher histone retention, thus affecting the protamine DNA packaging, and subsequently altering the imprinting process in some regions of sperm, thus compromising the activation of epigenetic programming following fertilisation. Such conditions may lead to an increased incidence of imprinting disorders in offspring (Dada *et al.*, 2012). A recent proteomic study of mature human sperm identified at least 6198 sperm proteins of which 581 were sperm nucleoproteins (Amaral *et al.*, 2014). Interestingly, 56% of these nucleoproteins are potentially involved in epigenetic activity, including chromosome organisation, chromatin organisation, protein-DNA complex assembly, DNA packaging, gene expression, transcription, chromatin modification and histone modification (Castillo *et al.*, 2014). These enriched biological processes in relation to sperm nucleoproteins include two major epigenetic events; (1) chromatin remodelling during nucleohistone-nucleoprotamine transition and (2) histone modification (Carrell, 2012;

Schagdarsurengin *et al.*, 2012) which is essential for the involvement of protamine and other nucleoproteins in the setting of epigenetic marks (Castillo *et al.*, 2014).

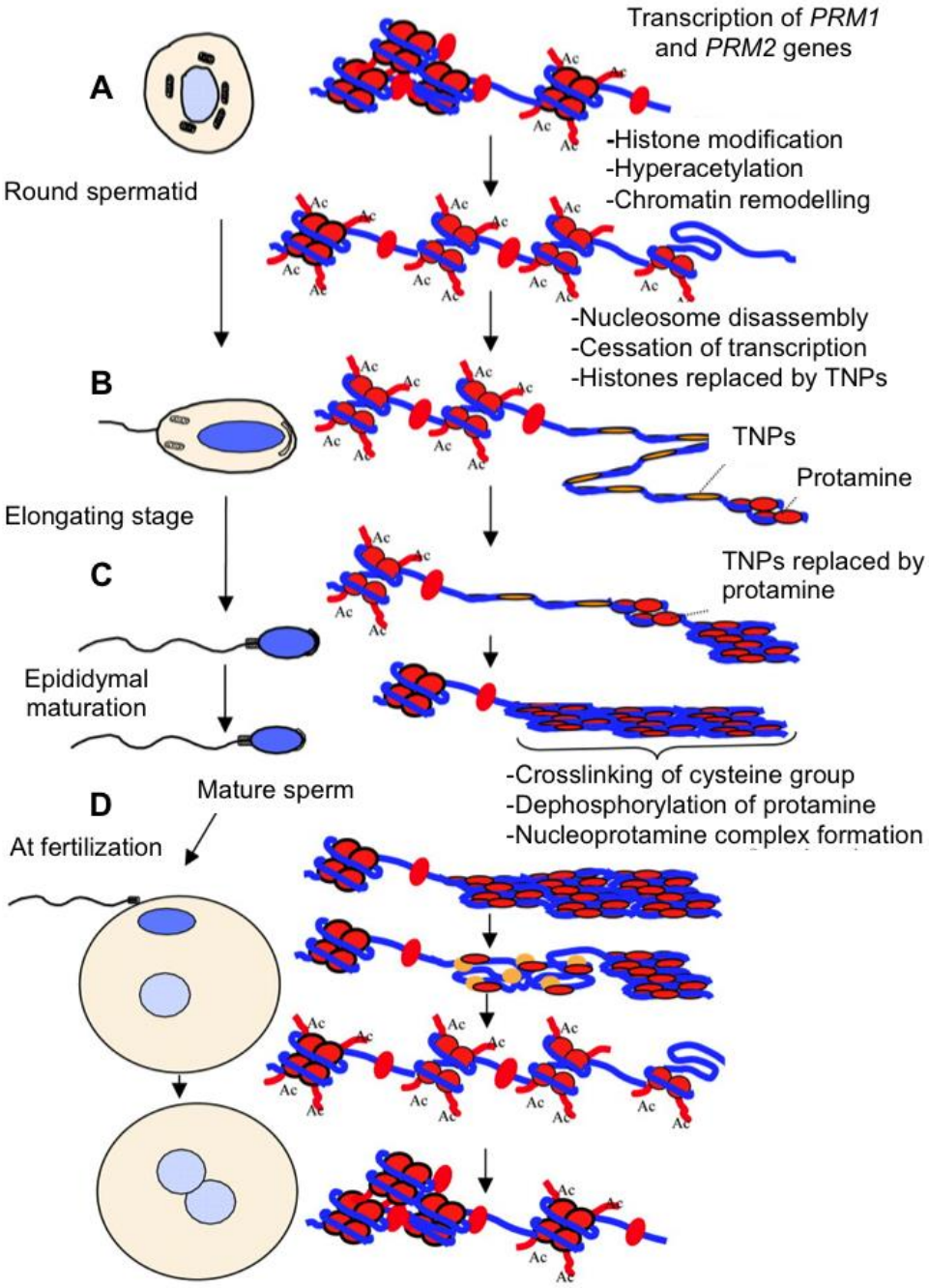


Figure 1.9: The nucleohistone/nucleoprotamine mechanism during spermatogenesis and spermiogenesis**. (A)** At the round spermatid stage, chromatin structure is organized by histones in a manner similar to that somatic cells. The *PRM 1* and *PRM 2* genes are actively transcribed and stored in round spermatid cells. Proceeding towards the beginning of spermiogenesis, the histones undergo hyperacetylation and modification. **(B)** The nucleosomes actively disassemble and the helical DNA is unwound by topoisomerase enzyme II. Transcriptional activity is terminated at this stage and histones are rapidly replaced by TNPs in chromatin-DNA. **(C)** Towards the end of spermiogenesis, the TNPs are actively displaced by protamines. At the epididymal maturation stage, the protamine-DNA complex is further stabilized by the formation of disulphide bridges. **(D)** Following fertilisation, disulfide bonds are reduced, protamines are disassociated and DNA unpacks preparing for reorganization into nucleosome structure. Histones are indicated in red, DNA in blue, transition proteins (TNPs) in orange oval and protamines in red ovals. Adapted and modified from Oliva (2006).

Since the sequential processes of histone/protamine displacement are essential, especially in the formation of the DNA-protamine complex structure which provides protection to sperm nuclear DNA, a robust and reliable chromatin remodelling mechanism is required from the start (Oliva & Castillo, 2011; Dada *et al.*, 2012; Gannon *et al.*, 2014). Reports show that in human sperm, the P1/P2 ratio is usually equivalent to approximately 1.0 or falls within a narrow range of 0.8 - 1.2 (Oliva, 2006). Alterations in P1/P2 ratio are often associated with male infertility (Balhorn *et al.*, 1988; Belokopytova *et al.*, 1993; de Yebra *et al.*, 1998; Carrell & Liu, 2001; Mengual *et al.*, 2003; Torregrosa *et al.*, 2006; de Mateo *et al.*, 2009; Simon *et al.*, 2011; Rogenhofer *et al.*, 2013), or abnormalities in the mechanisms controlling chromatin remodelling or histone modification, thus leading to possible errors during spermatogenesis (Dada *et al.*, 2012).

1.3.2 Altered protamine ratio, DNA integrity and male infertility

While the P1/P2 ratio remains relatively consistent in fertile human sperm, several studies have reported altered ratios, and/or absence of P2, in sperm from infertile men. These changes have received much attention in terms of the potential clinical use of P1/P2 ratio as a biological tool for sperm selection in ART. This would be particularly prudent given that current semen analysis in clinics is only based upon a narrow range of criteria rather than the specific assessment of DNA quality, integrity and function.

Protamine abnormalities among infertile men were first identified in 1976 using acid-urea gel electrophoresis, a direct assessment specifically designed to evaluate protamine in sperm nucleoprotein extracts. The significance of this method is further discussed in Chapter 3 of this thesis. Interestingly, sperm from these patients only expressed histones, not protamine (Silvestroni *et al.*, 1976) indicating that the infertile status was related to the absence of protamine. A subsequent study reported significantly elevated levels of P1/P2

ratio in seven infertile men, along with poor sperm motility and low concentration (Balhorn *et al.*, 1988). Since elevation in P1/P2 ratio has been linked with reduced levels of P2 in infertile patients, this has generated interest as to whether P2 alone may play a significant role in determining the overall P1/P2 ratio (de Yebra *et al.*, 1993). Reduced or altered P2 expression leading to abnormalities in the P1/P2 ratio have been associated with poor semen parameters including reduced progressive motility, poor penetration ability, and abnormal sperm morphology (de Yebra *et al*., 1993; Carrell & Liu, 2001; Aoki *et al*., 2005a; Aoki *et al*., 2006a). The identification of elevated levels of P2 precursors with reduced levels of P2 among infertile men indicated that an abnormal P1/P2 ratio may represent abnormal P2 processing and may influence DNA compaction, leading to detrimental changes in semen quality parameters. (de Yebra *et al.*, 1998; de Mateo *et al.*, 2009). Alternatively, altered P1/P2 ratios may arise from defective histone replacement during spermiogenesis. Indeed, increased levels of histones have been reported in sperm from infertile men compared to fertile controls (Zhang *et al.*, 2006; Montellier *et al.*, 2013) and in some cases sperm from these patients exhibited abnormal head morphology (Blanchard *et al.*, 1990).

Several studies have associated abnormalities in P1/P2 ratio with increased levels of DNA fragmentation (Aoki *et al.*, 2005a; Torregrosa *et al.*, 2006; de Mateo *et al.*, 2009; Castillo *et al.*, 2011; Simon *et al.*, 2011; Kumar *et al.*, 2012). For example, increased levels of DNA fragmentation were detected in patients with low P1/P2 ratio (Aoki *et al.*, 2005a). Additionally, patients with low levels of either P1 or P2 (or both) showed significant elevation in DNA fragmentation compared to those with normal levels. Torregrosa *et al*. reported a positive correlation between the presence of P2 and increased DNA fragmentation (Torregrosa *et al.*, 2006). Furthermore, an immunofluorescence study of individual sperm viability, followed by measurement of protamine abnormalities and DNA fragmentation, indicated significant heterogeneity in protamine expression, and low P1/P2 ratio, associated

with increased DNA fragmentation and poor cell viability (Aoki *et al*., 2006a). More recent studies have provided further evidence to link increased levels of DNA fragmentation in protamine deficient sperm from infertile men (Castillo *et al.*, 2011; Simon *et al.*, 2011; Kumar *et al.*, 2012). Interestingly, comparative studies of DNA fragmentation rate in cryopreserved sperm from eleven different mammals indicated that specific structural attributes of P1 and P2, such as the presence of cysteine groups, correlated with chromatin stability and resistance to cryo-injury, indicating that the DNA integrity of mammalian sperm exhibiting a higher number of cysteine residues in the protamine sequence was unaffected following cryopreservation (Gosalvez *et al.*, 2011).

1.3.3 Altered protamine expression, mutation and polymorphisms

The marked alterations in P1 and P2 content in infertile males led investigators to focus upon mutation in the *PRM1* and *PRM2* genes (Belokopytova *et al.*, 1993; de Yebra *et al.*, 1993), especially considering that the absence of P2 in sperm from some mammals (eg. bull, ram and pig) appear to arise from a dysfunctional *PRM2* gene (Maier *et al.*, 1990; Balhorn, 2007). Although mutations in *PRM1* and *PRM2* genes are considered to be rare, single nucleotide polymorphisms (SNPs) in both *PRM1* and *PRM2* have been identified in infertile males (Tanaka *et al.*, 2003). Within a large study group of fertile and infertile men, four SNPs were identified in the coding region for *PRM1* and one SNP (*c*248*t*) in *PRM2* which led to the formation of a stop codon. The authors postulated that this type of mutation in *PRM2* would cause the premature termination of P2 mRNA, resulting in infertility (Tanaka *et al.*, 2003). Another study demonstrated a single SNP in the *PRM1* gene (*G*197*T*) causing the substitution of arginine for serine (R34S) in three infertile patients (Iguchi *et al.*, 2006). These changes would interrupt the arginine DNA-anchoring mechanism and additionally

create a new phosphorylation site for P1 leading to abnormalities in protamine packaging (Iguchi *et al.*, 2006; Jodar *et al.*, 2011).

It is transparent that protamine deficiency affects routine seminal parameters such of motility, morphology and concentration. A recent study reported higher sperm count and concentration in males possessing three SNPs: *PRM1* 230AA, and *PRM2* 298CC and 373CC. Males homozygous for these SNPs exhibited a two-fold higher sperm output compared to males without this haplotype (Tuttelmann *et al.*, 2010). Moreover, a group of Spanish patients exhibiting morphologically abnormal sperm possessed an SNP in the *PRM1* gene promoter region (-191 C>A) and an elevated P1/P2 ratio (Gazquez *et al.*, 2008; Jodar *et al.*, 2011). Similarly, another SNP in the *PRM1* promoter region was observed in four males with severe oligozoospermia (Ravel *et al.*, 2007). Although the consequence of this mutation remains undefined, the authors suggest that interruption within this specific region of DNA as a result of this SNP may lead to abnormal P1 expression (Ravel *et al.*, 2007). While male infertility arising from differential protamine genotype (Tuttelmann *et al.*, 2010), and disparities in the open reading frames of the *PRM1* and *PRM2* genes, lead to abnormal protamine expression (Jodar *et al.*, 2011), the incidence of these conditions are rarely considered as a predominant factor for male infertility, especially considering the clear heterogeneity between individuals and populations.

Potential errors incurred during protamine transcription, translation and post-translation modification are highly likely to cause alteration in P1 and P2 expression (Carrell *et al.*, 2007). For example, the cyclic adenosine monophosphate (cAMP) response element modulator (CREM), a transcriptional regulator, is involved in the transcription of several genes during the post-meiotic phase of spermatogenesis, including *PRM1* and *PRM2* (Ha *et al.*, 1997). A transgenic mouse featuring deletion of the CREM activator, CREM (ACT), exhibited significantly lower sperm counts, and severe head malformations, caused by

defective chromatin compaction and acrosome formation (Kotaja *et al.*, 2004). Thus, defects in sperm arising during the transcription and translation of protamine may lead to abnormal protamination, further affecting nuclear condensation, and consequently compromising their functional ability to fertilise an oocyte.

Recent studies have demonstrated significant correlation between alterations in protamine messenger ribonucleic acid (mRNA) ratio and abnormal seminal parameters (Depa-Martynow *et al.*, 2012) in relation to male infertility. Protamine genes are actively transcribed and stored in round spermatids and translated during the elongating spermatid stage (Carrell *et al.*, 2007). Defective protamine transcriptional activity may thus contribute to abnormal protamination and/or alteration to P1/P2 ratio upon completion of spermiogenesis and maturation (Depa-Martynow *et al.*, 2012; Rogenhofer *et al.*, 2013).

1.3.4 Protamine alteration, IVF potential and iatrogenic damage

Variability in the protamine content of sperm from infertile patients undergoing infertility treatment is a critical factor to consider (Aoki *et al.*, 2006a), especially when several reports have demonstrated a positive fertilisation outcome in terms of embryo quality, implantation and pregnancy rates following IVF/ICSI using sperm from protamine-deficient patients (Carrell & Liu, 2001; Nasr-Esfahani *et al.*, 2004; Aoki *et al.*, 2005a; Tavalaee *et al.*, 2012b).

It has been demonstrated that protamine-deficient patients exhibit poor IVF outcome compared to ICSI, indicating the reduced penetration ability of sperm from such patients (Aoki *et al.*, 2006b). These findings also suggest that heterogeneity in the concentration of protamine is a useful factor to consider when selecting sperm for ICSI with poor quality and DNA damage (Aoki *et al.*, 2006b). Sperm selected for ICSI based on basic morphology and quality may exhibit normal protamine expression, but this scenario is highly debateable since

there is no clear method of measuring levels of protamine in a single sperm, especially when poor seminal parameters observed among infertile patients have frequently been associated with abnormal protamine expression (Carrell *et al.*, 2007).

However, several reports have contradicted these observations. For example, alteration in P1/P2 ratio, characterised as either higher or lower to the normal range, were strongly associated with poor sperm motility, concentration and morphology (Torregrosa *et al.*, 2006; Castillo *et al.*, 2011; Nanassy *et al.*, 2011). ICSI outcomes from such sperm were low, indicating the importance of normal protamine content. In addition, sperm from mice exhibiting deletion of either P1 or P2 demonstrated higher rates of embryonic death following ICSI. These data indicate that both P1 and P2 are equally essential for normal embryonic development (Cho *et al.*, 2001; Cho *et al.*, 2003). Furthermore, several studies have shown that abnormal protamination, or altered P1/P2 ratio, in human sperm results in poor embryo quality with consequential reduction in fertilisation and pregnancy outcomes (Simon *et al*., 2011; Depa-Martynow *et al*., 2012).

The apparent correlation between altered P1/P2 ratio and various aspects of male infertility created discussion as to whether protamine measurement could facilitate the selection of the best sperm for ICSI, or serve as a biological indicator to predict the success of IVF/ICSI. More recently, the measurement of protamine mRNA ratios has been suggested as a beneficial way to evaluate the fertilisation potential of sperm for clinical application (Rogenhofer *et al.*, 2013). However such a technique does not represent a method for selecting a single sperm with optimal DNA integrity or protamine content for ICSI. The same study also demonstrated that protamine-deficient sperm from normozoospermic men exhibited poor fertilisation capability and provided further evidence that ICSI might be able to consistently overcome infertile cases associated with protamination defects (Rogenhofer *et al.*, 2013).

The significantly increased DNA fragmentation levels in sperm from protamine-deficient patients have been studied extensively (Castillo *et al.*, 2011; Simon *et al.*, 2011; Kumar *et al.*, 2013). Although protamine heterogeneity in sperm may overcome the selection of DNA-fragmented sperm (Aoki *et al.*, 2006a), the long term consequences of such sperm following ICSI, in terms of embryonic development and especially in the health of offspring remains undetermined. However, since protamine is proposed to be involved in regulating epigenetic events and the generation of imprinted genes (Oliva, 2006), the possibilities of transmitting genetic errors may be attributed to abnormal protamination and DNA fragmentation in sperm (Castillo *et al.*, 2014).

Sperm nuclear chromatin is a specialised structure (Castillo *et al.*, 2014) consisting of imprinting genes involved in transcriptional activities, thus indicating the fundamental role of the paternal genome for the developing embryo (Hammoud *et al.*, 2009). Sperm nuclear epigenetic marks are represented by several major mechanisms such as DNA methylation, histone modifications and chromatin-bound proteins, including protamines and 8% of histones remain bound to DNA following spermiogenesis (Castillo *et al.*, 2014). DNA methylation sets a checkpoint for genetic imprinting during spermatogenesis (Dada *et al.*, 2012) and is essential for normal embryonic development (Hammoud *et al.*, 2009). Therefore, the retention of histones in paternal genomic DNA can lead to histone/protamine transition abnormalities (Dada *et al.*, 2012), which could compromise normal embryonic development (Oliva, 2006).

Currently, concern has arisen concerning the efficiency of ART techniques, especially when the incidence of conditions such as autism has been reported in children born following IVF/ICSI (Sandin *et al.*, 2013b). In addition, autistic disorders in children born via ICSI using surgically-retrieved sperm might be at higher risk compared to offspring born via ICSI using ejaculated sperm (Sandin *et al.*, 2013b). Although this risk is thought to be relatively low,

several investigators have further suggested that the use of ejaculated sperm and IVF treatment would be a better option than ICSI (Mayor, 2013). Furthermore, an increased prevalence of imprinted gene disorders such as Angelman, Beckwith-Wiedemann and Silver-Russell syndromes in offspring following ART has also been reported (Amor & Halliday, 2008; Hiura *et al.*, 2012). Collectively, these findings indicate that the sperm plays a crucial role in delivering genomic paternal DNA into the oocyte and that the use of protamine-deficient sperm for ICSI may carry significant risk for the developing embryo (Dada *et al.*, 2012).

To date, most investigators have used acid-urea gel electrophoresis to evaluate protamine levels in fertile and infertile men. However, the clinical application of this technique to predict sperm with the best DNA has to be evaluated. Crucially, this existing technique fails to provide information on the localisation pattern of protamine in sperm. It is not yet known as to whether this parameter plays an equally important role as protamine level. The present study thus aimed to develop a novel immunofluorescent method to detect protamine in sperm and compare with the existing acid-urea gel electrophoresis method. A secondary objective was to investigate the potential effect of cryopreservation, a common ART technique, upon protamine level, ratio and localisation pattern in sperm. At present, there is no data available as to how common laboratory technique may influence this important protein in terms of its expression, structure, or function. This represents a significant shortfall in our current knowledge. These investigations are described in Chapters 2 and 3.

1.4 Phospholipase C Zeta (PLCζ), the oocyte activation factor

This thesis concerns the effects of ART upon two key proteins in sperm. In **Section 1.3** of this chapter, I introduced protamine and its essential role in maintaining sperm DNA integrity. The following section introduced a second sperm-specific protein, PLCζ, which plays a crucial role in activating the egg following gamete fusion.

1.4.1 Calcium oscillations and oocyte activation

Oocyte activation occurs following gamete fusion and involves alleviation of the oocyte from second meiotic arrest, cortical granule exocytosis, formation of the pronucleus and protein expression (Amdani *et al.*, 2013). Oocyte activation is initiated by the sperm upon fertilisation and involves coordinated changes in cytosolic Ca^{2+} concentration within the ooplasm (Stricker, 1999; Kashir *et al.*, 2013a) (**Figure 1.10**).

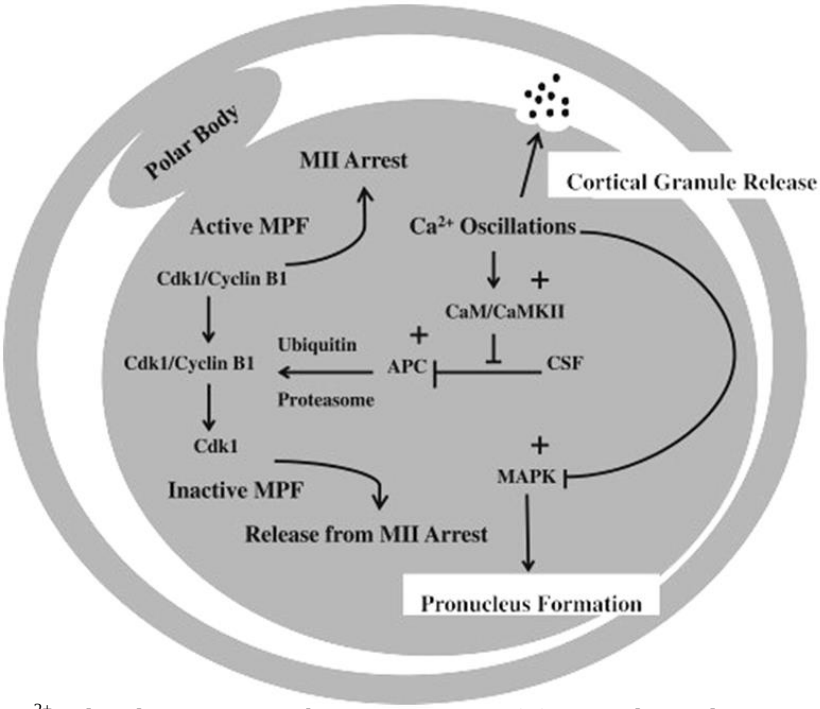


Figure 1.10: Ca^{2+} induced activation mechanism in oocytes. **(A)** Cortical granule exocytosis. **(B)** Activation of calcium/calmodulin-dependent protein kinase II (CaM/CaMKII) pathway. Upon activation, cytostatic factor (CSF) is inhibited followed by the release of anaphase-promoting complex/cyclosome (APC) which breaks down cyclin B1 in the maturation promoting factor (MPF) complex, composed of cyclin B1 and cyclin-dependent kinase 1(Cdk1). **(C)** The degradation of cyclin B1 causes inactivation of MPF and releases the oocyte from meiotic arrest. **(D)** Additionally, Ca^{2+} oscillation activates mitogen-activated protein kinase (MAPK) to facilitate pronuclei formation. Adapted and modified from Amdani *et al*. (2013).

The pattern of Ca^{2+} release varies across different animal species from a single large transient to a prolonged series of multiple oscillations (Stricker, 1999; Kashir *et al.*, 2013a). For example, in echinoderms, fish, and frogs, a single large Ca^{2+} transient is sufficient to activate the oocyte/eggs (Stricker, 1999; Kashir *et al.*, 2013a). However, in certain marine invertebrates, and in all mammals studied to date, the sperm induce a prolonged series of repetitive Ca^{2+} oscillations (Stricker, 1999; Amdani *et al.*, 2013; Kashir *et al.*, 2013a; Swann & Lai, 2013) (**Figure 1.11**).

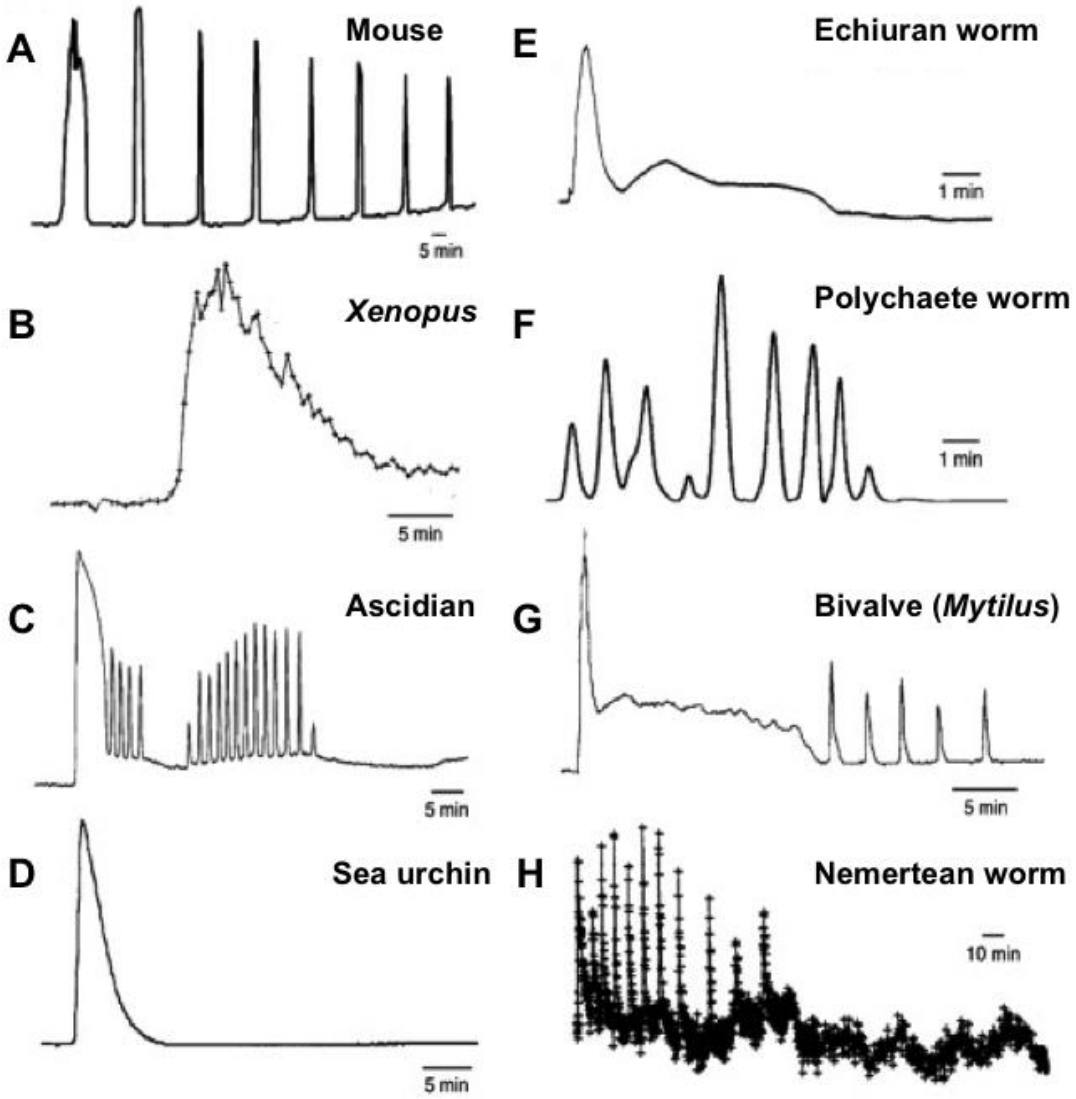


Figure 1.11: Single transient and multiple oscillations of Ca^{2+} release in oocytes and eggs from various species at fertilisation. **(A)** Mouse, **(B)** *Xenopus laevis*, **(C)** *Ciona savignyi*, **(D)** *Strongylocentrotus nudus*, **(E)** *Urechis caupo*, **(F)** *Chaetopterus pergamentaceus*, **(G)** *Mytilus edulis*, and **(H)** *Cerebratulus lacteus*. Adapted from Miyazaki and Ito (2006).

The production of single versus multiple transients may rely upon several oocyte characteristics, such as alleviation of the oocyte from meiotic arrest, the meiotic completion rate following fertilisation, and changes in levels of intracellular Ca^{2+} concentration during oocyte maturation (Stricker, 2006; Deguchi, 2007; Nader *et al.*, 2013). Differences in the Ca^{2+} release pattern across non-mammalian and mammalian species (Stricker, 1999; Kashir *et al.*, 2013a), explains the fundamental difference or species-specificity of the oocyte activation process among different organisms (Amdani *et al.*, 2013).

Three hypothetical models were originally put forward to explain how Ca^{2+} is released during oocyte activation: (**Figure 1.12A**); the Ca^{2+} conduit theory (Jaffe, 1983; Jaffe, 1991), (**Figure1.12B**); the sperm ligand and oocyte membrane receptor theory (Jaffe, 1990; Schultz & Kopf, 1995; Evans & Kopf, 1998; Parrington *et al.*, 2007); and the sperm factor theory (Swann *et al.*, 2006; Parrington *et al.*, 2007; Saunders *et al.*, 2007) (**Figure 1.12C**). A growing body of evidence now strongly advocates that the sperm factor theory (Amdani *et al.*, 2013) is the strongest model. Perhaps the most persuasive line of evidence is that ICSI technology directly concurs with the sperm factor model, since the microinjection of a single sperm into the ooplasm bypasses membrane fusion between the sperm and oocyte altogether, but still leads to successful activation (Parrington, 2001).

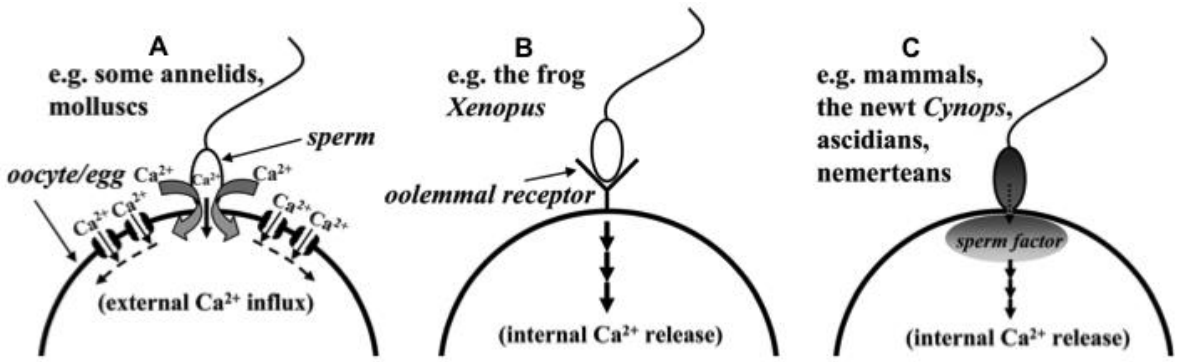


Figure 1.12: The three models proposed for fertilisation induced Ca^{2+} release. **(A)** Ca^{2+} conduit. **(B)** Sperm ligand and oocyte membrane receptor. **(C)** Soluble sperm factor. Adapted and modified from Kashir *et al.*

The first few lines of evidence which supports the sperm factor theory began when microinjection of either a single sperm or sperm soluble extracts into mammalian and some non-mammalian oocytes induced Ca^{2+} oscillation similar to those seen during fertilisation (Swann, 1990; Stricker, 1997; Fissore *et al.*, 1998). Studies also further indicated that the sperm factor was heat sensitive (Swann, 1990; Fissore *et al.*, 1998), and did not appear to be species-specific, since sperm extracts from a variety of species were able to elicit Ca^{2+} oscillation in mouse oocytes (Swann & Lai, 2013). Moreover, microinjection of mRNA isolated from spermatogenic cells into mouse oocytes triggered Ca^{2+} oscillations, while injection of mRNA from other tissues did not (Parrington *et al.*, 2000).

The acute rise in intracellular Ca^{2+} concentration within an activating oocyte is a direct result of increased levels of inositol 1,4,5-triphosphate (IP_3) which causes the release of Ca^{2+} from the endoplasmic reticulum (ER) mediated by the inositol 1,4,5-triphosphate receptor (IP_3R) (Stricker, 1999; Kashir *et al.*, 2013a).

A prolonged series of Ca^{2+} oscillations following ICSI have been demonstrated in both mouse and human oocytes (Tesarik & Sousa, 1994; Nakano *et al.*, 1997). In addition, the suppressed activity of IP_3R in mouse oocytes was observed following ICSI, indicating the IP_3 mediated Ca^{2+} release in the oocyte in response to this technique (Lee *et al.*, 2010). Collectively, these data clearly indicated the presence of a sperm-specific factor that initiated Ca^{2+} oscillations following the administration of a single sperm into the oocyte.

The identity of the sperm factor was finally resolved when mammalian sperm extracts triggered Ca^{2+} release with the production of IP_3, via the phosphoinositide (PI) specific (PLC) signalling pathway in intact sea urchin eggs and homogenates (Jones *et al.*, 1998; Jones *et al.*, 2000; Rice *et al.*, 2000; Wu *et al.*, 2001). In addition, the increasing levels of PLC enzymatic activity quantified in sperm extracts further confirmed that the identity of the soluble sperm

factor was likely to be a PLC protein (Jones *et al.*, 1998; Rice *et al.*, 2000). Existing PLCs isoforms such as PLCβ, γ, and δ were notably absent from sperm extract fractions (Wu *et al.*, 2001; Parrington *et al.*, 2002). Moreover, the microinjection of purified recombinant PLCβ1, γ1, γ2 or δ1 proteins into sea urchin eggs and egg homogenates failed to induce Ca^{2+} release (Jones *et al.*, 2000). Collectively, these observations led researchers to investigate the presence of a novel, uncharacterized isoform of PLC protein in mammalian sperm serving as the soluble factor responsible for Ca^{2+} release and subsequent oocyte activation.

1.4.2 Identification of PLCζ, the sperm-specific oocyte activation factor

A novel sperm-specific PLC protein that exhibited the specific characteristics of the soluble sperm factor responsible for oocyte activation was finally discovered by Saunders *et al*. (2002) using a mouse expressed sequence tag (EST) database and named phospholipase c zeta (PLCζ). PLCζ protein is testis-specific and the microinjection of mouse PLCζ complementary RNA (cRNA) into metaphase-II arrested mouse oocytes triggered Ca^{2+} oscillations similar to those seen during fertilisation (Saunders *et al.*, 2002). Quantitative analyses revealed that the amount of PLCζ in the fertilised mouse oocyte fell within an identical range to the concentration of PLCζ estimated to be expressed by a single sperm (Saunders *et al*., 2002). This finding further confirmed the physiological properties of PLCζ as the stimulant for Ca^{2+} induced oocyte activation (Saunders *et al.*, 2002). Moreover, the immuno-depletion of PLCζ from sperm extracts completely inhibited Ca^{2+}releasing ability, indicating the fundamental role of PLCζ as the sole sperm-specific factor responsible for oocyte activation (Saunders *et al.*, 2002). In addition, the microinjection of partially PLCζ-deficient sperm from transgenic mice triggered Ca^{2+} release in mouse oocytes which ended prematurely (Knott *et al.*, 2005).

The discovery of PLCζ in mouse sperm led to the subsequent identification of PLCζ in monkey and human sperm (Cox *et al.*, 2002), and a variety of other mammalian and non-mammalian species including chicken, medaka fish and quail (Coward *et al.*, 2005; Ito *et al.*, 2008; Mizushima *et al.*, 2009). It was therefore proposed that PLCζ represents a universal trigger for oocyte activation (Coward *et al*., 2005). Microinjection of PLCζ cRNA or recombinant PLCζ protein from these species into mouse oocytes universally triggered Ca^{2+} oscillations (Amdani *et al.*, 2013; Nomikos *et al.*, 2013a). Recently, a brain and ovarian form of PLCζ was identified in two puffer fish species (*Takifugu rubripes* and *Tetraodan nigroviridis*) (Coward *et al.*, 2011). PLCζ cRNA from pufferfish failed to induce Ca^{2+} oscillations following injection into mouse oocytes, suggesting that the oocyte activation mechanism in puffer fish differs from many other species. The precise roles of the brain and ovarian PLCζ isoforms have yet to be determined (Coward *et al.*, 2011; Amdani *et al.*, 2013).

1.4.3 Structure and function of PLCζ

To date, thirteen isoforms of PLC enzymes have been identified and classified accordingly to their specific structure and function: PLCdelta (PLCδ 1, 3, 4), PLCbeta (PLCβ 1-4), PLCgamma (PLC γ1, γ2), PLCzeta (PLCζ), PLCepsilon (PLCε) and PLCeta (PLCη 1 and 2) (Gresset *et al.*, 2012; Kadamur & Ross, 2013). The common structural domains present in all PLCs are the X and Y catalytic active site at the central domain which is flanked with a single PKC-homology type II (C2) domain at the C-terminus and four tandem elongating factor (EF) hand domains at the N-terminus (Saunders *et al.*, 2002). PLCζ (**Figure 1.13A**), shares 33% structural similarity with PLCδ1 (**Figure 1.13B**) (Kashir *et al.*, 2012a), and exhibits a unique distinctive feature over other PLCs in that it lacks pleckstrin homology (PH) and Src homology (SH) domains. Consequently, PLCζ represents the smallest isoform

among the PLC family with a molecular mass of 70kDa in the human and 74kDa in the mouse (Saunders *et al.*, 2002).

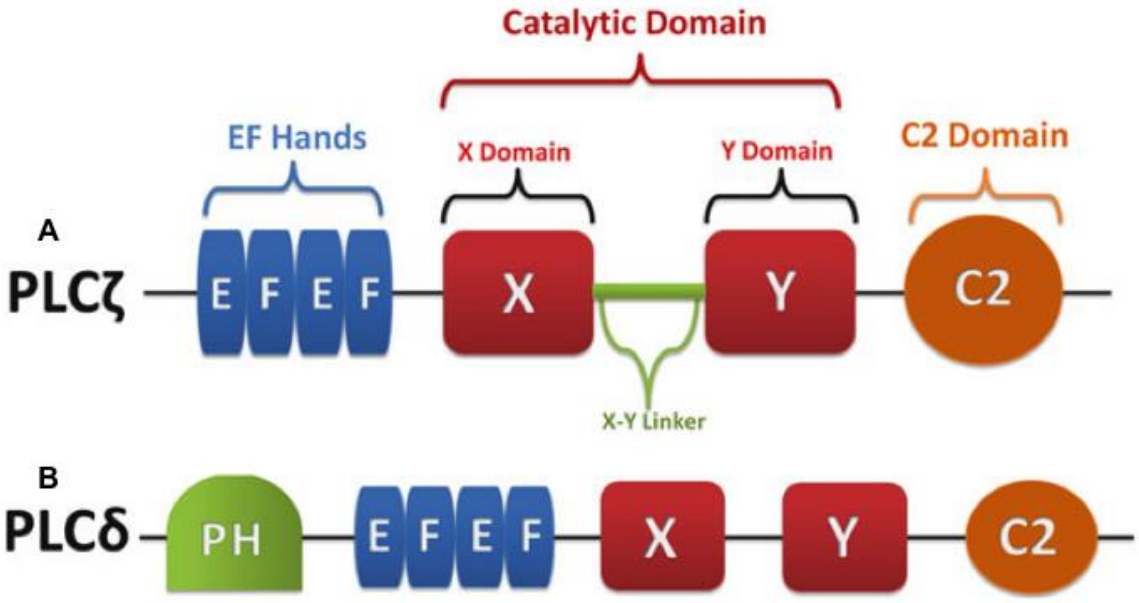


Figure 1.13: Typical protein domain structure of (**A**) phospholipase C zeta (PLCζ) in comparison to its closest homologue (**B**) phospholipase C delta (PLCδ). Adapted from Kashir *et al*. (2012a).

Despite being the smallest PLC isoform and lacking a PH domain, PLCζ confers the highest degree of sensitivity in triggering Ca^{2+} release within an oocyte as compared to other PLCs (Nomikos *et al.*, 2013b). For example, PLCζ activity is efficient at a concentration of just 40fg per egg (Saunders *et al.*, 2002; Nomikos *et al.*, 2005), while PLCδ1 is only able to induce Ca^{2+} oscillations at a concentration of 1pg per egg (Kouchi *et al.*, 2004) indicating that PLCζ exhibits very high potency to induce Ca^{2+} oscillations in mouse oocytes (Nomikos *et al.*, 2013b). In addition, several studies had indicated that PLCζ protein might undergo proteolysis and post-translation modification in order to establish enzymatic activity (Kurokawa *et al.*, 2007; Cooney *et al.*, 2010). While future studies may focus upon understanding the specific mechanism and investigating the post-translation activity of PLCζ, each structural domain of PLCζ demonstrates vital function in the induction of Ca^{2+}

oscillations leading to oocyte activation (Amdani *et al.*, 2013; Nomikos *et al.*, 2013b; Swann & Lai, 2013).

The X and Y catalytic domain is highly conserved and plays a major role in regulating PLCζ enzymatic activity (Swann *et al.*, 2006). Mutation of the X-Y domain failed to induce Ca^{2+} oscillations in mouse oocytes indicating the vital functional role of PLCζ in PIP_2 hydrolytic enzymatic activity (Saunders *et al.*, 2002; Nomikos *et al.*, 2012). The segment that connects the X and Y catalytic domain is refer to as the X-Y linker and is a key regulatory domain for targeting substrate. However, the linker region encompasses distinctive structural and biochemical features which exhibit different functional roles compared to other PLCs (Amdani *et al.*, 2013). The X-Y linker in PLCβ, γ, δ, ε η exhibits potent auto-inhibitory enzymatic regulation, due to its negatively-charged composition which may therefore impose electrostatic repulsion upon binding to PIP_2 (Nomikos *et al.*, 2011a; Gresset *et al.*, 2012). However, the PLCζ linker region is highly basic and positively-charged thus providing the potential to anchor negatively-charged PIP_2 substrate via the formation of electrostatic bonds (Nomikos *et al.*, 2011a).

The identification of a nuclear localisation signal (NLS) within the X-Y linker region of mouse PLCζ further indicated the importance of this region in modulating enzymatic function (Yoda *et al.*, 2004; Saunders *et al.*, 2007). Initially, it had been proposed that the NLS mediated PLCζ translocation to the pro-nuclei during zygotic interphase in mouse oocytes, which prevented interaction between PLCζ and PIP_2, subsequently terminating Ca^{2+} oscillations (Larman *et al.*, 2004; Yoda *et al.*, 2004). While a nuclear translocation mechanism was clearly demonstrated in mouse, a distinct NLS has yet to bee identified in human PLCζ.

A distinctive characteristic of PLCζ compared to other PLCs is its high sensitivity to Ca^{2+}. PLCζ exerts 100-fold higher sensitivity to Ca^{2+} as compared to its closest homologue, PLCδ1 (Swann & Yu, 2008; Nomikos *et al.*, 2012). Such an increased level of Ca^{2+} sensitivity present in PLCζ protein is attributed to the four tandem EF hands at the N-terminus of the protein (Nomikos *et al.*, 2013b). Experimental evidence demonstrated that deletion of one or both EF hand domains abolished Ca^{2+} oscillations in mouse oocytes (Kouchi *et al.*, 2005; Nomikos *et al.*, 2005). In addition, mutation within the PLCζ EF hands domain resulted in the interruption of nuclear translocation in mouse oocytes, suggesting the possible involvement of the EF hands in nuclear translocation (Kuroda *et al.*, 2006).

The C2 domain represents a typical feature among the PLC family, and is predominantly involved in membrane trafficking (Lomasney *et al.*, 2012). In PLCδ1, the C2 domain binds to phosphatidylserine (PS) for enzymatic regulation (Lomasney *et al.*, 2012; Nomikos *et al.*, 2012). Enzymatic activation in PLCβ is initiated following interaction of the C2 domain with the G-protein subunit, $G\alpha_q$ (Gresset *et al.*, 2012). Interaction between the PLCζ C2 domain and phosphatidylinositol 3-phosphate (P1-3P) leads to the reduction of PIP_2 hydrolytic activity (Kouchi *et al.*, 2005). As shown in PLCδ1, the X-Y linker folds to the EF hands and C2 domain to form a catalytic core (Kuroda *et al.*, 2006). Similarly, computerised three-dimensional modelling of the putative PLCζ structure suggests that PLCζ may undergo functional conformational changes by interaction between the EF hands and C2 domain, subsequently revealing the NLS (**Figure 1.14**) (Kuroda *et al.*, 2006). Alternatively, the C2 domain may associate PLCζ with targeting membrane-bound PIP_2 to initiate oocyte activation (Swann *et al.*, 2006).

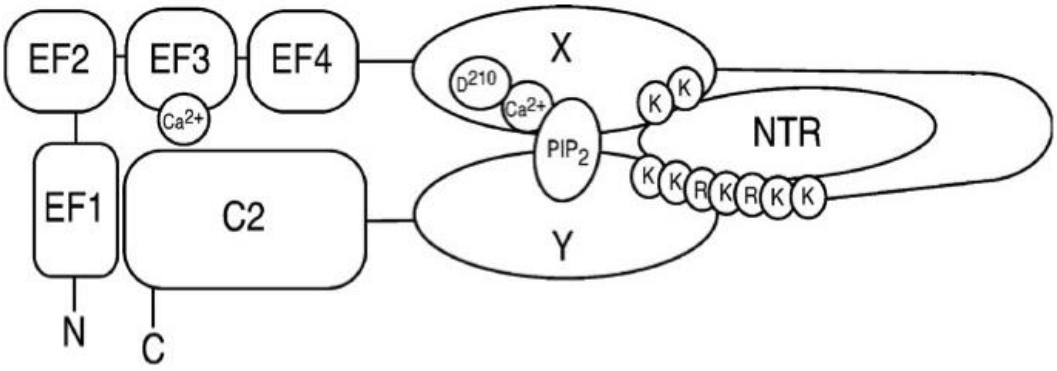


Figure 1.14: Illustration of a wild-type phospholipase C zeta (PLCζ) protein model showing folding between the EF-hand domain and C2 domain to establish functional conformation for Ca^{2+} oscillation-inducing ability and nuclear translocation ability. Adapted and modified from Kuroda *et al*. (2006).

The current hypothesis of how PLCζ induces Ca^{2+} oscillations in oocytes is illustrated in **Figure 1.15**. It is postulated that following gamete fusion, the sperm delivers PLCζ protein, which diffuses from the sperm head into the ooplasm and hydrolyses vesicular-bound phosphotidylinositol 4, 5-biphosphate (PIP_2) to produce IP_3. The generation of IP_3 subsequently activates IP_3R localised on the surface of the endoplasmic reticulum resulting in the release of Ca^{2+} (Nomikos *et al.*, 2013b; Swann & Lai, 2013).

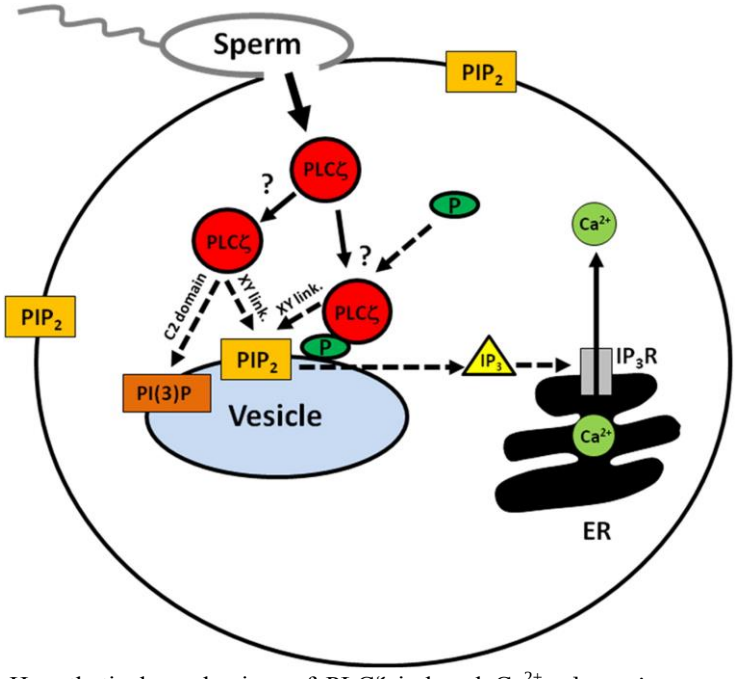


Figure 1.15: Hypothetical mechanism of PLCζ induced Ca^{2+} release in oocytes. Following gamete fusion, sperm releases phospholipase C zeta (PLCζ) into the oocyte, which subsequently hydrolyses phosphatidylinositol biphosphate (PIP_2) bound to intracellular vesicular membrane to generate inositol triphosphate (IP_3) from the endoplasmic reticulum (ER) internal store. PLCζ may specifically target the vesicular membrane via interaction of the C2 domain with either phosphotidyl inositol-3-phospahte [PI(3)P] or an unknown membrane protein (P). Adapted from Nomikos *et al*. (2013b).

An additional role for the C2 domain may involve of anchoring PLCζ to a target receptor located on intracellular PIP_2 -containing vesicles to regulate IP_3 mediated Ca^{2+} rise in the ooplasm (Nomikos *et al.*, 2013b). While the PH domain in other PLCs exerts a fundamental role in binding to the plasma membrane and PIP_2, the enzymatic regulatory mechanism in PLCζ in response to the absence of the PH domain remains debatable. However, evidence indicates that the X-Y linker and C2 domain are predominantly involved in targeting substrate to promote enzymatic activity (Nomikos *et al.*, 2011b).

While the role of PLCζ in the oocyte activation mechanism is well characterized, the oocyte itself may exhibit certain degrees of association with PLCζ in order to induce Ca^{2+} oscillations (Swann *et al.*, 2006; Kashir *et al.*, 2010), particularly when, PLCζ remains in an inactive form in the sperm prior to its release into the oocyte. Although the precise mechanism underlying the inactive state of PLCζ in sperm has yet to be determined, the involvement of an oocyte-borne factor in targeting PLCζ to its substrate for the activation of enzymatic activity is highly apparent (Amdani *et al.*, 2013; Nomikos *et al.*, 2013b).

In relation to this hypotheses, it has been suggested that endogenous oocyte PLCs such as PLCβ, γ, and δ maybe involved in the Ca^{2+} oscillation regulatory mechanism during oocyte activation (Saunders *et al.*, 2002). The first evidence for the involvement of oocyte-borne factors in relation to Ca^{2+} oscillatory activity was recently reported, whereby the reduced expression of oocyte PLCβ altered the Ca^{2+} oscillation profile during fertilisation (Igarashi et al., 2007). Alternatively, oocyte cytoplasmic maturation may be a key fundamental mechanism in determining the regular/normal Ca^{2+} release required for successful fertilisation, since fertilised immature mouse oocytes exhibited Ca^{2+} transients with lower amplitude (Eppig *et al.*, 1994). Moreover, oocyte maturation is associated with cytoplasmic changes in relation to the Ca^{2+} release mechanism which includes endoplasmic

reticulum (ER) re-organisation, increased Ca^{2+} concentration and IP_3R sensitivity, and mitochondrial distribution and function (Cheung *et al.,* 2000).

Mammalian cell expression studies provide additional evidence corresponding to the requirement of an oocyte factor to activate PLCζ enzymatic activity. Adenosine triphosphate (ATP)-induced Ca^{2+} release in PLCζ expressed Chinese Hamster Ovary (CHO) cells, did not exhibit Ca^{2+} release, although PLCζ enzymatic activity was observed in PLCζ-transfected CHO cell lysates. However, microinjection of PLCζ transfected CHO cell lysate into mouse oocytes triggered Ca^{2+} oscillations suggesting the presence of an oocyte factor that was able to activate the functional ability of PLCζ (Phillips *et al.,* 2011). A similar investigation using Human Embryonic Kidney (HEK) cells was reported by Kashir *et al*. (2011b). HEK cells expressing human PLCζ protein lysates triggered Ca^{2+} oscillations leading to pronuclei formation in mouse oocytes. However, the onset of Ca^{2+} oscillations was delayed by up to an hour following injection of cell lysates thus indicating the requirement for PLCζ to undergo post-translational modification or interactions with an unknown oocyte factor in order to activate functional ability (Kashir *et al.*, 2011b).

Further confirmation that an interactive oocyte factor may play an essential role in triggering Ca^{2+} release was evident following a PLCζ sub-cellular localisation study in mouse oocytes using Venus- or- YFP tagged PLCζ fusion protein or indirect immunofluorescence. PLCζ was shown localise at small intracellular vesicles that were distributed evenly within the ooplasm but not the plasma membrane. Interestingly, this study also identified intracellular vesicles containing PIP_2, indicating that PLCζ enzymatic activity may occur uniformly across the ooplasm (Yu *et al.,* 2012). Furthermore, the C2 domain of PLCζ may target phosphatidylinositol 3-phosphate (P1-3P) or an unidentified oocyte membrane protein located on intracellular vesicles, subsequently modifying PLCζ to an active state in order to gain functional ability (Nomikos *et al.,* 2013b) (**Figure 1.15**). Although the sub-cellular

vesicular localisation of PLCζ in the mouse oocyte and the potential involvement of an oocyte factor able to bind PLCζ have been hypothesized, the precise factor involved has yet to be identified, in mouse or any other oocyte.

1.4.4 Localisation of PLCζ in sperm

ICSI using isolated sperm heads triggered oocyte activation in mouse oocytes and thus indicated that the sperm factor was a cytosolic protein localised entirely within the sperm head (Perry *et al.*, 1999). PLCζ has been demonstrated to localise in distinctive regions within the sperm head, across a variety of mammalian species. For example, in non-capacitated mouse and bull sperm, PLCζ was predominantly localised in equatorial and post-acrosomal regions (Yoon & Fissore, 2007). Another study identified acrosomal and post-acrosomal localisation within mouse and hamster sperm (**Figure 1.16**) (Young *et al*., 2009). Following capacitation, the acrosomal region diminished while the post-acrosomal regions became more distinct (Young *et al.*, 2009) suggesting that PLCζ may undergo changes or translocation during capacitation and the acrosome reaction and may be involved in different functional roles aside from oocyte activation. PLCζ was subsequently identified in the acrosomal and post-acrosomal regions of pig sperm and an additional population was demonstrated in the tail (Nakai *et al.*, 2011).

In human sperm, PLCζ is localised in acrosomal, equatorial and post-acrosomal regions, with the equatorial population representing the predominant pattern (Grasa *et al.*, 2008). This study also showed that these patterns appeared to be localised in various combinations within fertile human sperm without any consistent pattern (Grasa *et al.*, 2008). As seen in mouse and hamster sperm, human PLCζ was predominantly localised within the equatorial and post-acrosomal regions following capacitation (Grasa *et al.*, 2008). More recently, significant variability among these three distinctive PLCζ patterns was reported.

Indeed seven various combinations of these three PLCζ localisation patterns were identified in this particular study, which represented the largest study at the time: acrosomal, equatorial, post-acrosomal, acrosomal + equatorial, acrosomal + post-acrosomal, equatorial + post-acrosomal, and all three patterns together (Kashir *et al.*, 2013b). In a more detail study, Kashir *et al*., (2013b) later re-emphasized the findings of Grasa *et al*., (2008) and found that the equatorial and post-acrosomal population was the most prevalent (**Figure 1.16**).

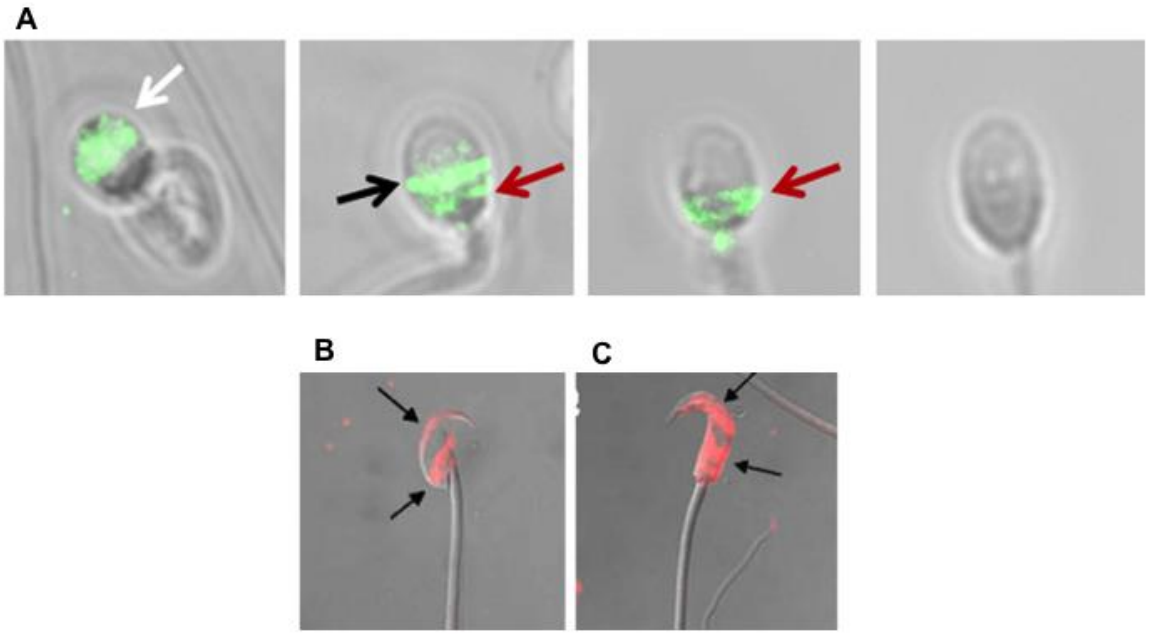


Figure 1.16: Immunofluorescent microscopy images of phospholipase C zeta (PLCζ) localisation patterns**. (A)** Human sperm exhibiting PLCζ at the acrosomal region represented with a white arrow, equatorial and post-acrosomal region indicated with black and red arrows respectively and in the post-acrosomal region indicated with a red arrow. The far right panel shows sperm devoid of PLCζ. **(B)** Mouse sperm and **(C)** hamster sperm both showing PLCζ localisation in the acrosome and post-acrosomal region as indicated by the black arrows. Adapted and modified from Young *et al*. (2009) and Kashir *et al*. (2013b).

While the precise localisation of PLCζ remains undetermined, existence of the multiple PLCζ populations suggests that PLCζ may participate in differential functional roles besides oocyte activation, such as capacitation and the acrosome reaction during fertilisation (Fujimoto *et al.*, 2004; Grasa *et al.*, 2008; Bi *et al.*, 2009; Young *et al.*, 2009; Kashir *et al.*, 2013b). Moreover, the acrosomal population of NYD-SP27 protein (an isoform of PLCζ) in human and mouse sperm was suggested to represent a de-capacitation factor (Bi *et al.*, 2009).

The specificity of the PLCζ antibody used in these studies and the different localisation patterns identified have been questioned particularly with regards to the polyclonal nature of existing antibodies. However, the consistency of the PLCζ localisation patterns observed in human sperm clearly supports the existence of multiple populations (Grasa *et al.*, 2008; Kashir *et al.*, 2013b).

Whilst emerging data clearly support the role of PLCζ as the sperm-borne mammalian oocyte activation factor (Amdani *et al.*, 2013), it is apparent that oocyte activation ability alone may not be sufficient to completely justify PLCζ function in relation to the sperm factor theory (Swann & Lai, 2013). This is because several other candidates have been proposed, such as citrate synthase and post-acrosomal sheath WW domain binding protein (PAWP). Both of these proteins have been suggested to be involved in mammalian oocyte (Harada *et al.*, 2007; Wu *et al.*, 2007). However, it is apparent that these candidates are not able to elicit the physiological Ca^{2+} rise seen during mammalian fertilisation (Swann & Lai, 2013) and clinical evidence to support such hypotheses is insufficient (Amdani *et al.*, 2013). For example, a recent study proposed a contradictory theory postulating that PLCζ is not the sperm-borne candidate responsible for oocyte activation (Aarabi *et al.*, 2012). These authors proposed that PLCζ is not a cytosolic sperm factor, and is, infact localised at the surface of the post-acrosomal region in mouse/bull sperm and over the entire head of human sperm. This study clearly disputes the previous findings of PLCζ as a cytosolic protein with distinct localisation within the sperm head. In addition, this study also suggested that PLCζ is a protein secreted by the epididymis and does not diffuse into the ooplasm following gamete fusion (Aarabi *et al.*, 2012). However, the validity of these findings remains highly debatable since the sample size utilised in this particular study was small, and antibody specificity was questionable.

While mounting molecular and clinical evidence continue to support the fundamental role of PLCζ as the most reliable candidate responsible for oocyte activation, the generation of PLCζ knockout mice and purified active human recombinant PLCζ protein may further strengthen the credibility of PLCζ as the sole sperm-borne oocyte activation factor (Amdani *et al.*, 2013). The lack of supporting evidence from the clinical arena represents a significant drawback to the indications made by Aarabi *et al*. (2012).

1.4.5 Clinical links between PLCζ and male infertility

The prevalence of infertility has led to significant investment in an attempt to provide the best levels of clinical care for couples seeking fertility treatment (Gnoth *et al.*, 2003). As a direct result, ART now accounts for 1.5% and 7% of all child births in the UK (Zegers-Hochschild *et al.*, 2009; Ramadan *et al.*, 2012) and developed countries (Nasr-Esfahani *et al.*, 2010) respectively. While ICSI remains highly effective, particularly with respect to treating male factor infertility, and delivers success rates in the range of 70%-80% (Heindryckx *et al.*, 2005; Palermo *et al.*, 2009), approximately 1 - 5% of ICSI cases fail, accounting for approximately 1200 cases annually in the UK alone (Ramadan *et al.*, 2012). Oocyte activation deficiency (OAD) is thought to be the principal cause for ICSI failure (Heindryckx *et al.*, 2008; Kashir *et al.*, 2010), although oocyte factors such as incomplete cytoplasmic maturation and deficiencies in the PI signalling pathway are also thought to play contributory roles.

Currently, OAD is treated by the application of artificial oocyte activators (AOA) such as Ca^{2+} ionophores, ethanol, or strontium chloride (Yanagida *et al.*, 2008; Taylor *et al.*, 2010; Vanden Meerschaut *et al.*, 2014). While AOAs appear to be a reliable source of treatment for OAD cases, concern has been raised with regards to the potential detrimental effects from such chemicals upon embryo viability, function and offspring health, especially

considering the cytotoxic, mutagenic and teratogenic properties of Ca^{2+} ionophores (Nasr-Esfahani *et al.*, 2010). Critically, Ca^{2+} ionophores induce a single Ca^{2+} transient rather than a series of periodic oscillations. This is worrying given that Ca^{2+} oscillations are vital in regulating oocyte activation and gene expression in the early embryo (Heytens *et al.*, 2009). By eliciting a single Ca^{2+} transient, Ca^{2+} ionophores may alter progression of the cell cycle and gene expression leading to abnormal embryonic development (Dupont *et al.*, 2010).

Given the fundamental role of PLCζ in oocyte activation, it follows that abnormalities in the expression, localisation, structure or function, of this critical sperm protein are likely to result in OAD or infertility (Ramadan *et al.*, 2012). Yoon *et al*. were the first to demonstrate that sperm from ICSI failure patients exhibited abnormal expression and localisation of PLCζ and failed to elicit Ca^{2+} oscillations following injection into mouse oocytes (Yoon *et al.*, 2008). Interestingly, these authors further demonstrated that oocyte activation ability could be rescued by the co-injection of mouse PLCζ mRNA, proving that PLCζ represents a valuable therapeutic agent (Yoon *et al.*, 2008). Further studies demonstrated that globozoospermic sperm exhibited abnormal levels and localisation patterns of PLCζ, and in some cases, were completely devoid of PLCζ (Heytens *et al.*, 2009; Taylor *et al.*, 2010). Two mutations of the gene encoding PLCζ were later identified in a single non-globozoospermic infertile patient. Occurring in the X and Y catalytic domains respectively, these two single point mutations (H398P and H233L, respectively) led to structural anomalies in protein structure, resulting in perturbation in binding properties and deficiency in the ability to induce Ca^{2+} oscillations in the ooplasm (Heytens *et al.*, 2009; Kashir *et al.*, 2012b) (**Figure 1.17**). While current human genetic screening for PLCζ mutation has focused upon exonic regions of the PLCζ gene (Heytens *et al.*, 2009; Kashir *et al.*, 2012b) it is imperative to incorporate other genomic regions such as the introns and promoter which may exert a fundamental role in PLCζ gene expression. Recently, two genetic mutations (-456G>A and +65T>C) in the PLCζ gene

promoter region were identified in Chinese Holstein bulls, which were associated with alterations in semen quality, such as volume of semen, sperm motility and morphology (Pan *et al.*, 2013). However, this study failed to expand their findings in relation to Ca^{2+} releasing ability and specific expression levels of these PLCζ variants. It would, therefore be interesting for future research to focus upon screening introns and the promoter region of the human PLCζ gene (Amdani *et al.*, 2013). Most recently, Kashir *et al*. (2013b) reported that while total levels of PLCζ in individual human sperm varied significantly, the proportion of sperm exhibiting PLCζ in fertile controls males was significantly higher than that of OAD patients (Kashir *et al.*, 2013b). Collectively, these findings strongly support the fact that PLCζ-deficiency is intimately linked to OAD and that PLCζ provides substantial potential as a novel therapeutic agent (Nomikos *et al.*, 2013a; Swann & Lai, 2013).

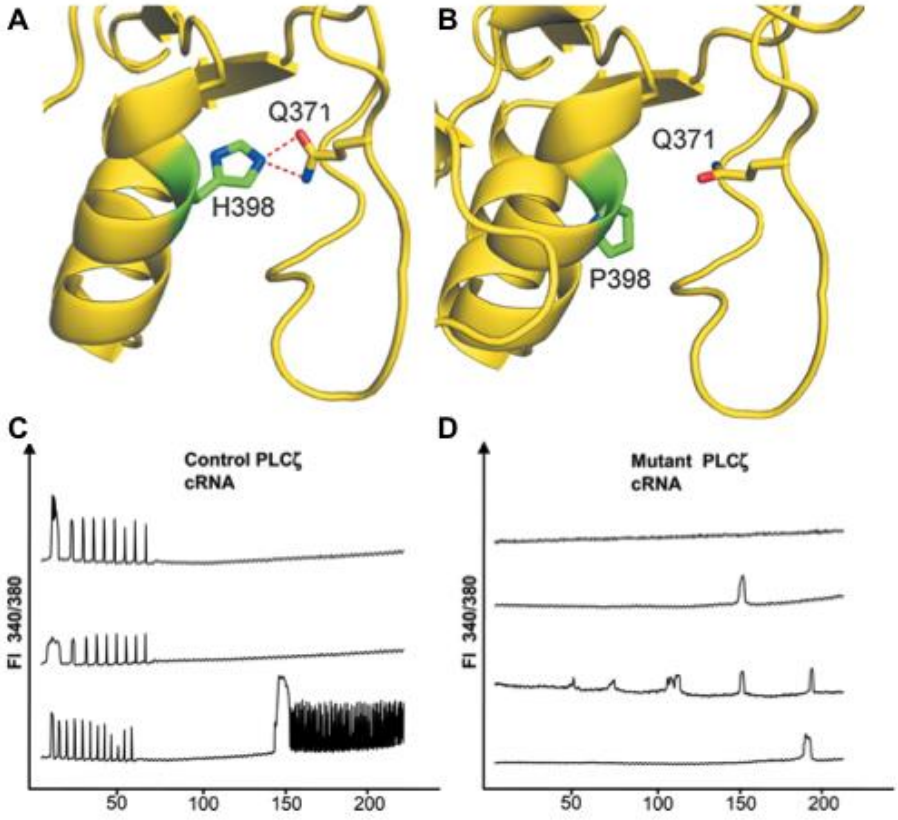


Figure 1.17: The first phospholipase C zeta (PLCζ) point mutation identified within the Y-catalytic domain by Heytens *et al*. (2009). **(A)** PLCζ model illustrating histidine residue at position 398 (H398) with side-chain-side-chain hydrogen bond, **(B)** Mutant PLCζ, proline substitution at position 398 (P398) without side-chain-side-chain hydrogen bond. **(C)** Microinjection of wild type PLCζ and **(D)** mutant PLCζ into mouse oocyte and subsequent Ca^{2+} oscillation profile. Adapted from Heytens *et al*. (2009).

1.4.6 PLCζ as a therapeutic agent for OAD

Mounting clinical evidence currently shows clear links between PLCζ abnormalities and OAD suggesting the potential therapeutic use of PLCζ to overcome OAD and ICSI failure. For example, microinjection of PLCζ cRNA into human oocytes generated Ca^{2+} oscillations leading to successful embryonic development up to the blastocyst stage (Rogers *et al.*, 2004). In addition, Yoon et al. (2008) demonstrated that the co-injection of PLCζ mRNA with ICSI failed PLCζ-deficient human sperm into mouse oocyte triggered normal Ca^{2+} oscillations (Yoon *et al.*, 2008). However, the clinical utilisation of PLCζ cRNA is not feasible due to uncontrollable transcriptional activity within the oocyte, which may compromise embryonic viability and function (Rogers *et al.*, 2004; Ozil *et al.*, 2006). Alternatively, the development of pure, active human recombinant PLCζ protein (hrPLCζ) may serve as an appropriate clinical therapeutic agent replacing the use of AOAs in the treatment of PLCζ-deficient infertile men. The generation of hrPLCζ has been highly challenging over recent years, and the initial endeavour to purify hrPLCζ was demonstrated by Kashir *et al*. (2011b). This study showed successful expression of active hrPLCζ using a mammalian cell line which, following microinjection into mouse oocytes, triggered Ca^{2+} oscillations similar to those seen during normal fertilisation. However, this particular hrPLCζ was not applicable therapeutically since PLCζ was present in non-purified mammalian cell lysates (Kashir *et al*., 2011b). Further experiments from other groups generated purified hrPLCζ expressed from bacterial cell lines. Yoon *et al*. (2012) were the first to synthesize an active purified protein. Microinjection of this protein induced Ca^{2+} oscillations in both mouse and human oocytes, however the Ca^{2+} oscillations profile observed in human oocytes appeared abnormal. In addition, the excessive concentration of protein required to stimulate Ca^{2+} oscillation rendered this protein unusable clinically (Yoon *et al.*, 2012). More recently, a Nus-A fusion protein tagged purified hrPLCζ was produced, which subsequently evokes Ca^{2+}

oscillations similar to those observed during fertilisation. Microinjection of this protein rescued oocyte activation ability in mouse oocytes which had previously been microinjected with mutant hrPLCζ (Nomikos *et al.*, 2013a) (**Figure 1.18**). Collectively, these data indicated that PLCζ is a potent therapeutic biological tool which could be implemented clinically. However, these findings urgently need to be extrapolated to a much large number of human oocytes in order to establish a standard protocol for clinical utilisation.

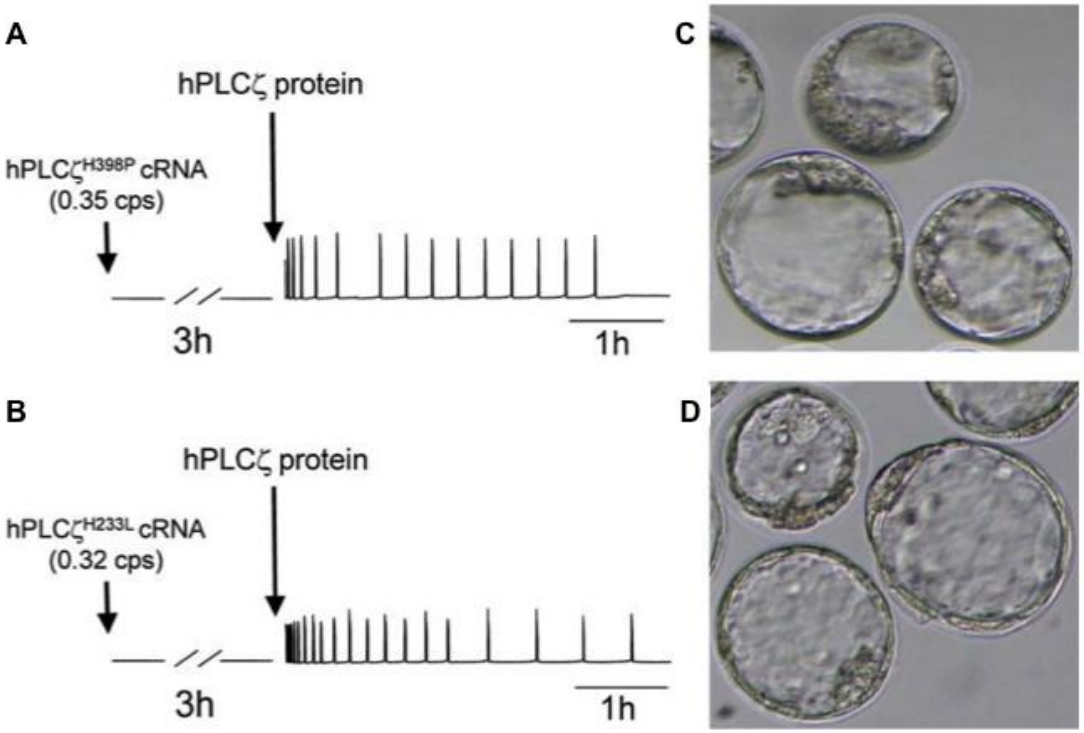


Figure 1.18: Failed oocyte activation following microinjection of mutant human phospholipase C zeta (PLCζ), subsequently rescued by microinjection with recombinant wild type human PLCζ protein. **(A)** Microinjection of mutant PLCζ ^{H398P}, **(B)** PLCζ ^{H233L} and subsequently injection with hPLCζ. **(C)**, **(D)** Micrographs representing mouse embryos at blastocyst stage following 96 hours post-injection with hPLCζ. Adapted from Nomikos *et al*. (2013a).

1.4.7 PLCζ as a prognostic/diagnostic marker

Apart from aiding as a therapeutic agent, PLCζ may also serve as a diagnostic or prognostic marker to indicate fertilisation ability. Currently, basic seminal parameter analysis is the routine clinical technique used to facilitate best sperm selection based upon morphology and motility (Amdani *et al.*, 2013; Kashir *et al.*, 2013b). However such methods of sperm selection do not completely eliminate abnormalities in sperm that may compromise the ability to fertilise since many patients still encounter unexplained fertilisation failure (Amdani *et al.*, 2013). This may suggest that additional biological makers may be required to assist the selection of sperm based on functional ability (Barratt *et al.*, 2011).

In addition to basic semen analyses, quantitative PLCζ immunofluorescence analysis may provide an informative biomarker in order to predict oocyte activation ability (Kashir *et al.*, 2013b). For example, the proportion of sperm exhibiting PLCζ was significantly higher in semen subjected to density gradient washing (DGW), (a routine clinical technique used to select best quality sperm) compared to sperm from raw semen. Such finding enhances the opportunity to select sperm exhibiting the best oocyte activation ability (Kashir *et al.*, 2011a). PLCζ mRNA is another quantitative measure which can be employed to compare oocyte activation ability between sperm from ICSI fail and fertile control men. However, the application of mRNA technology for such analyses may not be readily available in most fertility units (Aghajanpour *et al.*, 2011) as such screening may not be cost effective.

Advancement in ICSI has led to the implementation of intracytoplasmic morphologically selected sperm injection (IMSI) via motile sperm organelle morphology examination (MSOME). Successful pregnancy and childbirth from a 100% globozoospermic patient following IMSI without AOA application was reported by Sermondade *et al.* (2011).

In this study, MSOME technology enabled the selection of sperm which exhibited a distinctive acrosomal bud (Sermondade *et al.*, 2011).

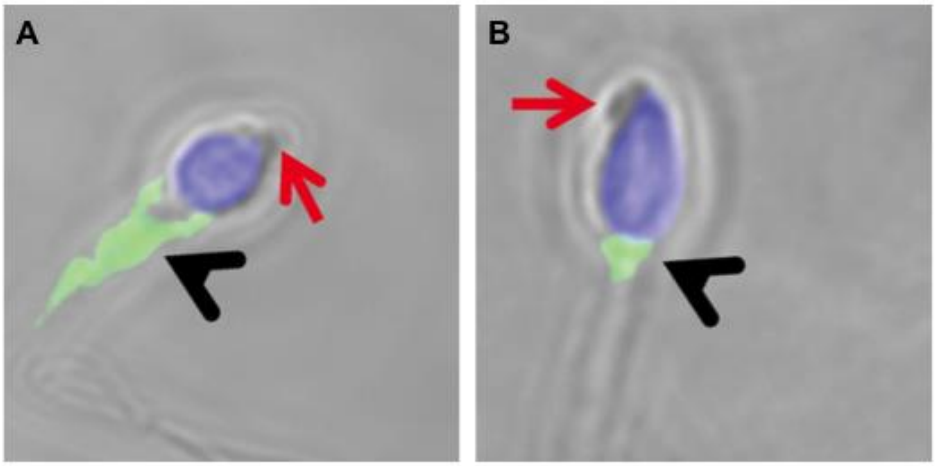


Figure 1.19: Immunofluorescent images of novel phospholipase C zeta (PLCζ) localisation pattern in motile sperm organelle morphological examination (MSOME)-selected globozoospermic sperm exhibiting an acrosomal bud (indicated by red arrow). **(A), (B)** Green fluorescence showing PLCζ localisation in the mid piece as indicated by black arrow head. Adapted from Kashir *et al*. (2012c).

Further investigation of this patient revealed that sperm possessing the acrosomal bud also exhibited a novel localisation pattern of PLCζ in the sperm head and mid-piece region (**Figure 1.19**). Moreover, sperm selected via MSOME from this globozoospermic patient exhibited higher levels of PLCζ as compared to non-MSOME selected sperm. In addition, total levels of PLCζ in globozoospermic sperm exhibiting an acrosomal bud did not show significant differences to sperm from control subjects (Kashir *et al.*, 2012c).

More recently, particle image velocimetry (PIV) technology was used to measure rhythmic cytoplasmic movement in activating oocytes in order to predict the viability of zygote development and thus helps to select the best embryo for intra-uterine transfer (Ajduk *et al.*, 2011). A strong correlation between cytoplasmic movement and Ca^{2+} oscillation profile in activating oocytes was observed, suggesting that the specific frequency of cytoplasmic Ca^{2+} oscillations delivered by PLCζ may influence rhythmic cytoplasmic

movement (Swann *et al.*, 2012). Hence, the application of PIV analyses clinically may be useful to indirectly monitor Ca^{2+} release within activating human oocytes and predict zygote viability.

1.4.8 Current concerns and outstanding issues

It remains largely unknown as to whether human PLCζ is susceptible to laboratory treatments and the artificial environment associated with ART. However, in a preliminary study, Kashir *et al*. (2011a) demonstrated that routine cryopreservation significantly reduced the total level of PLCζ in four out of seven fertile donors, via suspected freeze/thaw damage to the sperm membrane (Kashir *et al*., 2011a). The potential impact of other fundamental ART techniques, such as PVP treatment, remains untested. The use of PVP during ICSI is attracting increasing attention in terms of its potential to exert detrimental effects upon gametes and embryos. It has already been established that prolonged exposure of sperm to PVP can cause ultra-structural damage to the sperm membrane, nucleus, chromatin and may also induce the acrosome reaction (Strehler *et al.*, 1998; Gomez-Torres *et al.*, 2007; Kato & Nagao, 2012). It is therefore logical that any process that exerts a disruptive effect upon the sperm plasma membrane may lead to the translocation or suppression of key sperm proteins, including the oocyte activation factor (Dozortsev *et al.*, 1997). While PVP plays a critical role in regulating the volume of fluid injected into oocyte during ICSI (Kato & Nagao, 2012), there are significant concerns that non-degradable PVP may remain in the oocyte following injection, thus increasing susceptibility to zygotic damage (Jean *et al.*, 2001). Of more concern, however, is the fact that immobilisation of sperm prior to ICSI appears to influence the onset of Ca^{2+} oscillatory activity (Yanagida *et al.*, 2001), and consequently lead to abnormal embryonic development (Ozil *et al.*, 2006). Given the increasing reliance of ART upon ICSI, and the critical role played by PLCζ in the initiation of oocyte activation, it is

imperative that we understand how laboratory techniques associated with ART may influence the expression or functional ability of PLCζ.

1.5 Iatrogenic damage incurred to gametes during ART

One potential underlying factor to low ART success rate which has drawn recent attention is the potential for iatrogenic (clinician/procedure-induced) damage incurred to gametes and embryos while performing routine ART protocols which is quite often associated with processing gametes in an artificial environment (Agarwal *et al.*, 2006; Matsuura *et al.*, 2010; Yelumalai *et al.*, 2012). Following removal from their natural environment, gametes and embryos are generally manipulated and pre-treated by exposure to various mechanical and/or chemical treatments prior to IVF/ICSI in an artificial laboratory environment. The artificial manner in which gametes are introduced during ART represents a significant deviation from that which occurs naturally.

1.5.1 Cryopreservation of sperm

Cryopreservation is a routine technique considered fundamental to the field of ART (Di Santo *et al.*, 2012; Valcarce *et al.*, 2013). This technique permits the prolonged storage of gametes and embryos by preserving biological properties and quality (Jain & Paulson, 2006), for subsequent use during IVF or ICSI (Valcarce *et al.*, 2013). Sperm cryopreservation involves the freezing and storage of semen in liquid nitrogen at -196°C, where at this extreme temperature, the biological activities of sperm become arrested. Hence, sperm cryopreservation is pivotal for male patients undergoing medical procedures or surgical treatment which have the potential to affect their sterility (Paoli *et al.*, 2014). Cryopreservation also serves as a reliable tool for men with severe seminal abnormality conditions such as azoospermia or oligospermia, especially in cases where only a single spermatozoon is produced (Wang *et al.*, 2013). Thus, for men with such a condition

following Testicular Sperm Retrieval (TSR), cryo-storage prevents the need for repetitive biopsies (Donnelly *et al.*, 2001a; Donnelly *et al.*, 2001b). While cryopreservation of sperm plays an essential role in ART (Gosden, 2011), especially in cancer patients about to undergo chemotherapy or radiotherapy (van Casteren *et al.*, 2008), the potential detrimental effect of cryopreservation upon sperm structure and function is unavoidable (Zribi *et al.*, 2012).

The adverse effect of human sperm cryopreservation is multi-factorial (Gadea *et al.*, 2011), and frequently associated with cellular damage resulting from intracellular ice crystal formation, cold shock, osmotic stress and cell shrinkage (Muldrew & McGann, 1990; Watson, 2000; Zribi *et al.*, 2012). Although the specific physiological process of cryopreservation with relation to promoting detrimental effects upon sperm is poorly understood, it is commonly known that cell membranes are temperature sensitive and that rapid freeze/thaw processes affect membrane fluidity leading to cell injury, organelle and mitochondrial dysfunction (Watson, 2000). Therefore, it is feasible that such mechanisms may compromise sperm structure and functional ability, thus reducing the success of fertilisation outcome (Gandini *et al.*, 2006).

Previous studies concerned the motility of cryopreserved sperm and tested ability to fertilise mature oocytes (Hammerstedt *et al.*, 1990). However, it is widely reported that cryopreservation can cause aberrant effects upon seminal parameters such as motility, morphology, viability, and DNA integrity (Isachenko *et al.*, 2004b; Zribi *et al.*, 2010; Zribi *et al.*, 2012; Paoli *et al.*, 2014). Cryopreservation may therefore induce significant effects upon sperm velocity distribution (Marcus-Braun *et al.*, 2004; Thomson *et al.*, 2009; Lee *et al.*, 2012; Satirapod *et al.*, 2012) and reduces fertilisation rate compared to fresh untreated sperm (Borges *et al.*, 2007). Although, cryopreservation is considered as a reliable clinical tool to preserve, protect and sustain the biological properties of sperm, the enhanced assessment of

cryopreservation effects upon sperm quality at the molecular level is essential in order to reassure clinicians that this process does not compromise embryo health or viability.

Subsequently, a variety of studies have shown the impact of cryo-injury upon sperm DNA integrity leading to fragmentation (Donnelly *et al.*, 2001a; Donnelly *et al.*, 2001b; Zribi *et al.*, 2012; Valcarce *et al.*, 2013). DNA fragmentation does not necessarily relate to reduced or compromised fertilisation capability. However, there is increased risk of transmitting a fragmented or damaged paternal genome into an oocyte, and the deleterious effect arising from such a condition upon fertilisation, normal embryonic growth and offspring is a major concern, especially when poor ART outcomes such as low fertilisation rates, low embryo implantation, miscarriage, and cancer have been associated with sperm DNA fragmentation (Lewis & Aitken, 2005; Fatehi *et al.*, 2006; Fernandez-Gonzalez *et al.*, 2008; Aitken *et al.*, 2009). In addition, sperm DNA damage has been linked with increased risk of pregnancy loss following IVF and/or ICSI (Zini *et al.*, 2008). Therefore the assessment of DNA fragmentation in cryopreserved sperm is vital (Meseguer *et al.*, 2008), especially in light of the mounting evidence postulating a strong link between DNA fragmentation and male factor infertility.

Over recent years, several techniques have been developed and used to evaluate DNA fragmentation in cryopreserved sperm such as terminal deoxynucleotidyl transferase mediated dUTP, nick end labeling assay (TUNEL) ((Thomson *et al.*, 2009; Zribi *et al.*, 2010; Zribi *et al.*, 2012), sperm chromatin structure assay (SCSA) (Gandini *et al.*, 2006; Evenson & Wixon, 2008; Evenson, 2013), sperm chromatin dispersion test (SCD) (Gosalvez *et al.*, 2010) and the comet assay (Donnelly *et al.*, 2001b; Ribas-Maynou *et al.*, 2014). The mechanism underlying DNA fragmentation during spermatogenesis and epididymal transit has been widely reported and six models have been proposed (**Figure 1.20**). DNA fragmentation has been attributed to apoptosis during spermatogenesis, incomplete chromatin and/or protamine

condensation during spermiogenesis, and oxidative stress induced by the release of reactive oxygen species (ROS) during post-testicular transport (Cocuzza *et al.*, 2007; Sakkas & Alvarez, 2010). ROS-induced oxidative stress is frequently related to abnormal semen parameters, poor sperm function (Moustafa *et al.*, 2004), and the pathophysiology of defective human sperm (Cocuzza *et al.*, 2007). The generation of ROS and subsequent oxidative damage in sperm has also been attributed to cold shock during cryopreservation (Wang *et al.*, 1997). Gadea *et al*. (2011) further reported that ROS generation in sperm is caused by lipid peroxidation mediated by sperm-bound polyunsaturated fatty acids. Consequently, future research should investigate DNA fragmentation and ROS in order to prevent the use of sub-optimal sperm in ART.

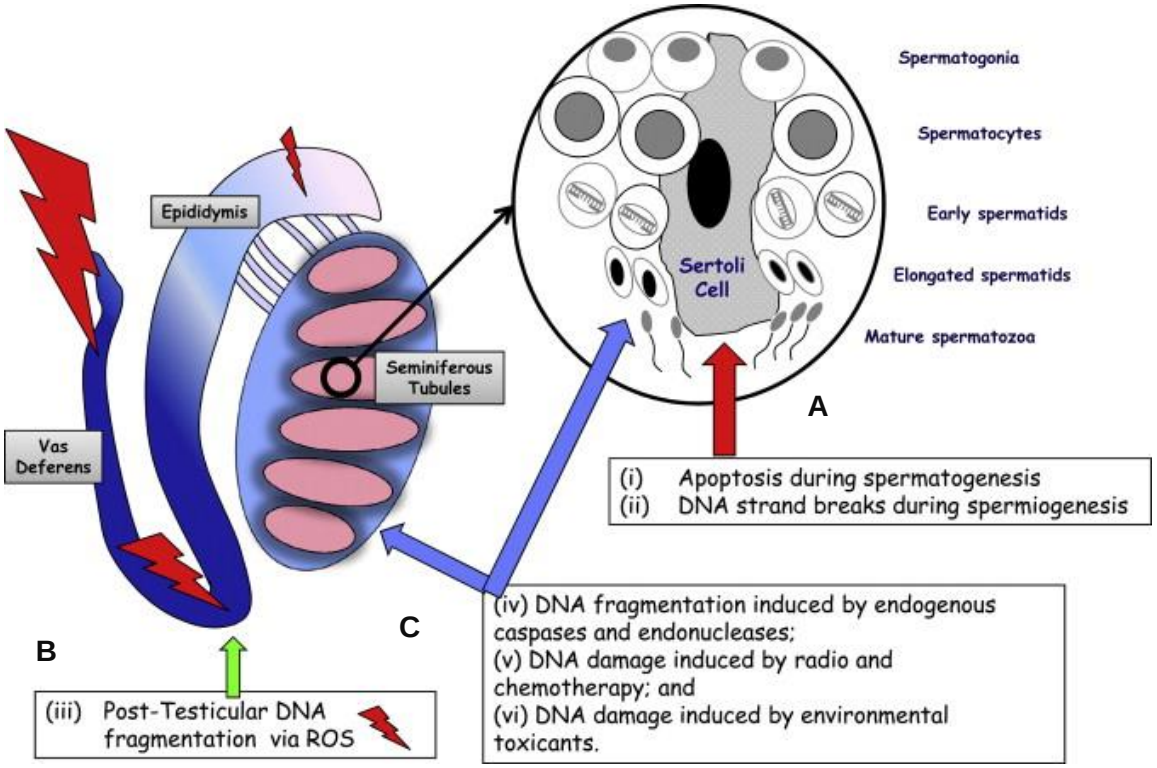


Figure 1.20: The six major models of DNA fragmentation occurring during sperm production and maturation. **(A)**: (i) Occurrence of apoptosis during spermatogenesis. (ii) DNA breaks during spermiogenesis. **(B)**: (iii) DNA fragmentation induced by oxidative stress via reactive oxygen species (ROS) during post-testicular transport through the epididymal passage. **(C)**: (iv) DNA damage caused by endonucleases and mutagens or during spermiogenesis. (v) DNA damage caused by radio/chemotherapy and (vi) effects caused by environmental toxicants. Adapted from Sakkas and Alvarez (2010).

While the effect of cryopreservation upon sperm structure and DNA integrity has been widely reported, very little is known about how cryo-injury might influence the levels and functional ability of sperm proteins. Early reports have demonstrated the detrimental effects of cryopreservation upon sperm-specific proteins that are crucial for fertilisation. For example, Cao *et al*. (2003) reported levels of heat shock protein 90 (H90) to be reduced significantly in human sperm following cryopreservation (Cao *et al.*, 2003). Similarly, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-Page) and Western blot analysis of freeze-thawed boar sperm further revealed significantly lower levels of H90 protein (Huang *et al.*, 1999). Additionally, surface proteins that act as mediators in the fertilisation pathway (McLeskey *et al.*, 1998) such as P25b were reduced in bull sperm after cryopreservation, with consequential reduction in fertilisation rates (Lessard *et al.*, 2000). Moreover, two dimension polyacrylamide gel electrophoresis (2D-PAGE) analysis of cryopreserved sea-bass sperm revealed degradation in 21 different proteins (Zilli *et al.*, 2005). Sperm glutathione, an antioxidant protein responsible for the prevention of oxidative damage in sperm was also significantly reduced in cryopreserved human sperm (Gadea *et al.*, 2011). More recently, Kashir *et al*. (2011a) demonstrated that cryopreserved sperm from healthy fertile men exhibited significantly reduced levels of PLCζ when compared to fresh sperm (Kashir *et al*., 2011a).

A recent proteomic study indicated variation in protein characteristics in cryopreserved sperm compared to fresh sperm obtained from fertile donors (Wang *et al.*, 2013). Interestingly, this study demonstrated significant alteration in the levels of 27 different sperm proteins following cryopreservation. In most cases, the levels of these crucial sperm proteins were significantly reduced in cryopreserved sperm compared to fresh sperm. However, several cases also demonstrated increased levels in certain sperm proteins following cryopreservation. Sperm proteins such as aconitate hydratase (ACO_2), tektin

(TEKT1), vimentin (VIM) and succinyl-CoA:3-ketoacid CoA transferase play a fundamental role in sperm motility, viability, acrosomal integrity, mitochondria membrane potential, capacitation, acrosome reaction and intracellular calcium concentration. Levels of these proteins were significantly reduced in cryopreserved sperm indicating degradation as a direct result of the freeze/thaw mechanism leading to dysfunction (Wang *et al.*, 2013). Such findings clearly demonstrated the detrimental effect of cryopreservation upon crucial sperm proteins which may compromise their fertilisation ability and thus influence ART success. Another proteomic study by Xiaoli *et al*. (2013) also demonstrated significant alteration in the levels of 41 different proteins in boar sperm responsible for premature capacitation, adhesion, energy supply and sperm-oocyte interaction. The authors proposed that alterations in 4 specific proteins [Superoxide Dismutase (SOD1), Triosephosphate Isomerase 1 (TPI1), Outer Dense Fibre 2 (ODF2) and A Kinase Anchoring Protein 3 (AKAP3)] following cryopreservation exerted detrimental effect upon sperm quality, thus reducing fertilisation ability (Xiaoli *et al.*, 2013).

Little is currently known as to how cryopreservation might influence protamine levels in human sperm. This is of great concern given the importance of protamine in maintaining DNA integrity. Several investigators have described the effects of freeze/thaw upon the structure of boar sperm nuclear DNA in relation to protamine and histone content (Flores *et al.*, 2008; Flores *et al.*, 2011). Mounting evidence has shown a strong link between protamine deficiency and infertility disorders in humans and rodents (Cho *et al.*, 2001; Cho *et al.*, 2003; Oliva, 2006). Protamine deficiency can be attributed to abnormal expression of P1 and P2 due to incomplete or abnormal histone/protamine transition during spermiogenesis (Oliva, 2006). For example, sperm from knockout mice model exhibiting protamine deficiency suffered significant spermatogenic abnormality and infertility (Cho *et al.*, 2001), DNA fragmentation, and embryonic death (Cho *et al.*, 2003). In addition, poor sperm

nucleohistone/protamine packaging is known to be induced by several factors including perturbation by endonucleases, mutagens, and ROS production (Sakkas & Alvarez, 2010), ultimately resulting in DNA fragmentation. While the effect of cryopreservation upon sperm integrity and DNA fragmentation had been widely reported, we know comparatively little about the possible links between cryopreservation and protamine levels and functional ability in human sperm. Such work may provide the opportunity to design a novel biological tool, which could be used to assess the nuclear integrity of thawed sperm prior to ART. Development of tests to evaluate an effect of any crucial sperm protein, besides protamine following cryopreservation would be worthy for the field of ART.

1.5.2 Immobilisation of sperm during ICSI

While the implementation of ICSI has been proven to be a very reliable technique, 1 - 5% of ICSI cases fail, accounting for approximately 1200 cases annually in the UK alone (Amdani *et al.*, 2013). Although sometimes associated with sperm expulsion during injection, inefficient injection technique, and abnormal sperm de-condensation, OAD is thought to be the primary factor underlying ICSI failure (Heindryckx *et al.*, 2008; Kashir *et al.*, 2010; Amdani *et al.*, 2013). It is also conceivable that ICSI failure may also be attributed, at least in part, to 'iatrogenic' (clinician-induced) damage incurred during the pre-treatment or manipulation of sperm (Binh *et al.*, 2009; Nakai *et al.*, 2011; Yelumalai *et al.*, 2012) or by sperm immobilisation techniques such as exposure of sperm to PVP prior to ICSI (Parmegiani *et al.*, 2010; Kato & Nagao, 2012; Parmegiani *et al.*, 2012). ICSI bypasses the acrosome reaction, an essential step required during natural fertilisation. Therefore, sperm plasma membrane damage via immobilisation may reduce integrity and increase permeability of the sperm plasma membrane to release the cytoplasmic matrix into an oocyte for activation and fertilisation (Dozortsev *et al.*, 1995; Palermo *et al.*, 1996a). However, extensive sperm

immobilisation may exert a certain degree of damage which may adversely compromise sperm function (Velaers *et al.*, 2012). Various sperm immobilisation techniques have been developed to increase sperm membrane permeability (Velaers *et al.*, 2012; Valcarce *et al.*, 2013) which in turn facilitates sperm handling and selection (Kato & Nagao, 2012) prior to ICSI treatment. Mechanical sperm immobilisation usually involves crimping or cutting the sperm tail in or below the mid-piece (Palermo *et al.*, 1996a), complete dissection of the sperm tail from the head and finally the utilisation of laser (Debrock *et al.*, 2003) or piezo-pulses (Yanagida *et al.*, 1999). PVP, a synthetic solution (Kato & Nagao, 2012) and hyaluronic acid (HA) (Parmegiani *et al.*, 2010), a physiological agent, are two examples of common chemical immobilisation techniques used for ICSI.

PVP has been used routinely in the field of ART, not only for sperm selection during ICSI, but also for oocyte culture media and cryopreservation (Kato & Nagao, 2012). The use of PVP in protein-free culture media permits IVF or *in vitro* maturation (IVM), embryo maturation, fertilisation and embryonic development to the blastocyst stage (Nagao *et al.*, 2010). The molecular weight of PVP is 10,000 Da and as such can play a role as potential cryoprotectant during cryopreservation (Suzuki *et al.*, 1995). Such protective effects arising from high molecular mass and viscosity may also prevent sperm nuclear de-condensation following ICSI, thus leading to a failure to form pro-nuclei. Furthermore, Percoll gradient centrifugation techniques, which utilise PVP in an inner column, enables the purification and selection of highly motile sperm (Nagao *et al.*, 2010). Moreover, PVP is used to prevent adherence of oocytes to plastic oocyte culture dishes (Hirao *et al.*, 2004). PVP increases the viscosity of sperm preparations, thereby reducing motility such that sperm can be manipulated and selected prior to ICSI (Van Steirteghem *et al.*, 1993; Martin, 2000).

Currently, more than 90% of published case reports and experimental studies supports the use of PVP during ICSI in fertility clinics (Kato & Nagao, 2012). However, a growing number of reports describe the detrimental effect of PVP upon sperm structure, nucleus, DNA integrity and function (Dozortsev *et al.*, 1995; Strehler *et al.*, 1998; Parmegiani *et al.*, 2010; Salian *et al.*, 2012). Sperm incubated in PVP prior to ICSI have been found to exhibit structural injuries such as damage to the sperm membrane, acrosome, head and nucleus (Kato & Nagao, 2012). Since ICSI treatment bypasses the acrosome reaction, several studies have postulated whether sperm plasma membrane damage (Dozortsev *et al.*, 1995) is required to facilitate the acrosome reaction during ICSI. Whether sperm plasma membrane damage is required for successful fertilisation via ICSI still remains vague (Velaers *et al.*, 2012), although the degree of adverse effect exhibited may compromise or alter functional ability of the sperm concerned. For example, when cultured in PVP, bovine sperm showed an increased rate of acrosome reaction compared to the control group (Kato & Nagao, 2009). This indicated that PVP appears to induce damage to the sperm plasma membrane, thus triggering the acrosome reaction (Kato & Nagao, 2012) which can cause the release of sperm cytosolic factors into the culture medium prior to ICSI.

Progressive sperm motility is one of the most vital criteria used to assess fertilisation capacity (WHO, 1999; WHO, 2010). Kato *et al.*, (2009) demonstrated that bovine sperm treated with PVP exhibited lower fertilisation rates compared to sperm incubated in a control medium and noted significant reduction in the rate of sperm penetration into the oocytes (Kato & Nagao, 2009). These findings signify that PVP may cause damage to the mitochondria, the source of ATP production to facilitate sperm tail movement (Amaral *et al.*, 2013) which could compromise progressive motility and influence interaction with oocytes leading to poor fertilisation rates. Worryingly, reports have shown that PVP exposure can also lead to nuclear deterioration in sperm (Strehler *et al.*, 1998) and significantly increased

levels of DNA fragmentation compared to sperm exposed to control medium or HA medium (Parmegiani *et al.,* 2010; Salian *et al.*, 2012). Collectively, these data suggest that the use of PVP may impose a high degree of adverse effects upon sperm structure, DNA integrity, and functional ability, which could also lead to subsequent fertilisation failure or abnormal embryonic development. While the adverse effects of PVP upon sperm is widely known, it would be interestingly to further investigate the consequence of such treatment upon specific proteins translated by the sperm proteome. Since, sperm nuclear deterioration following PVP treatment has already been demonstrated (Strehler *et al.*, 1998), it appears plausible that PVP may cause degradation to protamine, the sperm nuclear protein which plays a fundamental role in maintaining and protecting the sperm paternal genomic DNA. Thus far, no studies have demonstrated the effect of PVP upon levels of protamine in sperm, despite, several reports describing increased potential DNA fragmentation levels (Parmegiani *et al.*, 2010; Salian *et al.*, 2012) in PVP treated sperm. A key point to note, however is that DNA fragmentation in sperm has been strongly linked with abnormal protamination (Aoki *et al.*, 2005a; Castillo *et al.*, 2011; Garcia-Peiro *et al.*, 2011; Salehi *et al.*, 2013) suggesting that a link between PVP and protamine levels may be likely.

Interestingly, it is clear that sperm immobilisation techniques during ICSI can exhibit significant influence upon the onset of calcium (Ca^{2+}) oscillations in the oocyte (Yanagida *et al.*, 2001; Morozumi *et al.*, 2006). The pattern of Ca^{2+} oscillations evoked during mammalian fertilisation is essential for oocyte activation and normal embryonic development (Amdani *et al.*, 2013) and can exert a high degree of influence upon embryonic gene expression (Ozil *et al.*, 2006). Studies have shown that use of de-membranated and/or acrosome-less sperm (Morozumi *et al.*, 2006), which when further exposed to PVP initiated the acrosome reaction prematurely and enhanced pronuclear formation following ICSI (Kato & Nagao, 2009). Moreover, sperm incubated in PVP resulted in delayed initiation of Ca^{2+} oscillations in the

human oocyte following ICSI (Yanagida *et al.*, 2001). Mounting evidence supports the role of PLCζ as the sperm-borne factor responsible for inducing Ca^{2+} oscillations within the oocyte leading to oocyte activation (Amdani *et al.*, 2013). Thus, it is vital to investigate whether degradation of PLCζ protein in the sperm following PVP treatment also lead to abnormal Ca^{2+} release. In support of this hypothesis, the use of PVP for immobilising sperm tends to induce the acrosome reaction through cellular membrane disruption (Kato & Nagao, 2009) and leads to the liberation of important factors from the sperm cytosol. Most studies suggest that the co-injection of sperm with small volumes of PVP into the oocyte during ICSI may facilitate successful embryonic development and the birth of healthy offspring (Palermo *et al.*, 1996b; Kato & Nagao, 2012). However, in contrast, an earlier report indicated deleterious effects of PVP retention within oocytes (Jean *et al.*, 2001). PVP accumulation in the oocyte can cause abnormal pathological problems such as mucoid alteration as a result of interaction between PVP and oocyte cytoplasm. PVP engulfed in the ooplasm is indigestible by lysosome enzyme and would therefore remain in the oocyte. Such conditions may alter gene expression, embryonic development and genomic integrity leading to fertilisation failure or long-term effects upon the offspring health (Jean *et al.*, 2001). Moreover, PVP retained in the oocyte may also effect the interaction between PLCζ and oocyte-borne factors responsible for Ca^{2+} release.

Consequently, PLCζ deficiency in sperm treated with PVP, or retained PVP solution within the oocyte cytosol, may alter Ca^{2+} oscillatory pattern and resultant cytoplasmic movements in the early embryo thus compromising embryo development and viability. Since the detrimental effect of cryopreservation upon the levels of PLCζ in fertile sperm has already been demonstrated Kashir *et al.* (2011a), it would be interesting to investigate the abundance of PLCζ protein in sperm following PVP treatment.

This thesis collectively investigates the potential effects of ART laboratory procedures such as cryopreservation and the use of PVP as an immobilisation agent upon two vital sperm proteins, protamine and PLCζ. Both proteins are fundamental in the transmission of the paternal genome and activation of oocyte during fertilisation. Deficiency in these proteins arising from sperm manipulation and processing in an artificial laboratory environment may compromise functional ability, leading to detrimental effects upon fertilisation, abnormal embryonic development and alterations in gene expression during embryogenesis leading to genetic or epigenetic defects in offspring. Therefore, it is vital to first investigate whether ART laboratory protocols could alter these crucial proteins and compromise their functional ability for successful fertilisation following IVF or ICSI. The outcome of such investigations may allow improvement to current ART protocols, which may increase success rates. Moreover, the methodology used to screen protamine and PLCζ in this thesis may also represent potential clinical tools for sperm assessment to evaluate DNA integrity and oocyte activation ability.

1.6 General aims of thesis

1. Using a mouse model, Chapter 2 aimed to evaluate the effect of cryopreservation upon sperm concentration and motility, and DNA integrity using the SCD method in maturing epididymal sperm. Next, the total levels, localisation pattern and ratio of P1 and P2 were evaluated in non-cryopreserved and cryopreserved sperm. In addition, a novel immunofluorescent method was developed in order to determine protamine levels and localisation profile in sperm.

2. Chapter 3 aimed to investigate the detrimental effect of cryopreservation upon sperm parameters, total levels and ratio of P1 and P2, but with an established method, (acid-urea gel electrophoresis). The efficiency and protamine detection methods utilised in Chapters 2 and 3 were critically evaluated and compared.
3. Chapter 4 aimed to enhance our specific clinical knowledge of PLCζ by evaluating total levels, localisation pattern and the proportion of sperm exhibiting PLCζ in sperm from a large group of fertile controls and infertile patients using an immunofluorescent method. Several novel case studies associated with OAD, ICSI failure and PLCζ deficiency were identified and discussed.
4. In Chapter 5, the detrimental effects of PVP upon the total levels of PLCζ in sperm from a group of fertile controls and infertile patients were evaluated by immunocytochemistry.

Chapter 2

The effect of cryopreservation upon total levels of the sperm nuclear protein, protamine (P1 & P2) in mouse sperm:

A novel method for direct protamine quantification using immunofluorescent techniques

2.1 Introduction

During late spermatogenesis, the sperm nuclei are condensed and packed by P1 and P2 (Oliva, 2006; Balhorn, 2007; Francis *et al.*, 2014). The predominant function of protamine in terms of forming a highly compact package of sperm nuclear chromatin is to provide protection to paternal genetic material, and to preserve and maintain sperm DNA integrity. Furthermore, sperm nuclear condensation by P1 and P2 promotes formation of sperm hydrodynamic structure which is fundamental in determining optimal progressive motility in order to achieve the best fertilisation ability (Castillo *et al.*, 2014). Both P1 and P2 are present in most mammalian sperm, including humans, and mice. However, other species such as boar and bull exhibit only P1 in the sperm nuclei due to dysfunction of the P2 gene in these species (Balhorn, 2007). The organisation of P1 and P2 within sperm nuclei DNA is species-specific and in humans, the P1:P2 ratio is 1:1, whereas mouse sperm exhibits a P1:P2 ratio of 1:2 (Corzett *et al.*, 2002). The P1:P2 ratio in mammalian sperm, especially in humans, has been shown to play a fundamental role in determining sperm morphology, motility, DNA integrity, fertilisation capability, and has also been linked to embryonic development (Carrell & Liu, 2001; Cho *et al.*, 2001; Cho *et al.*, 2003; Aoki *et al.*, 2005a; Aoki *et al.*, 2006b; Castillo *et al.*, 2011; Garcia-Peiro *et al.*, 2011; Simon *et al.*, 2011; Rogenhofer *et al.*, 2013).

Mounting evidence indicates a clear association between alterations in protamine ratio and male factor infertility (Francis *et al.*, 2014). Altered P1:P2 ratios are frequently found in sperm from infertile men and these men generally exhibit abnormal seminal parameters such as low sperm motility, reduced sperm concentration or motile count and poor sperm morphology (Aoki *et al.*, 2005b; Aoki *et al.*, 2006a). Moreover, studies have shown that sperm from infertile men with an altered P1:P2 ratio exhibit lower sperm penetration assay (SPA) scores as compared to sperm from fertile men with normal protamine ratios.

Interestingly, sperm from these infertile men are able to successfully fertilise an oocyte following ICSI, but not after IVF treatment (Aoki & Carrell, 2003; Aoki *et al.*, 2005b). These findings indicate that abnormalities in protamine content may therefore have resulted in abnormal formation of the sperm hydrodynamic structure leading to poor motility, thus compromising fertilisation ability.

Routine sperm parameter analysis still serves as a useful sperm selection tool in terms of clinical application for IVF or ICSI, and to predict fertilisation ability (Larsen *et al.*, 2000; Guzick *et al.*, 2001; Keel, 2006). However, high variation in basic sperm parameters and the inherent heterogeneity of sperm has led to the development of additional biological tools with which to predict male infertility (Guzick *et al.*, 2001; Keel, 2006; Leushuis *et al.*, 2010; Barazani *et al.*, 2014), such as DNA fragmentation tests (Zini & Sigman, 2009). Such tests are considered to be beneficial, not only to evaluate fertilisation ability, but also to facilitate sperm selection (Practice Committee of the American Society for Reproductive, 2013) at optimal integrity, especially when sperm from infertile men are clearly associated with elevated levels of DNA fragmentation (Sakkas & Alvarez, 2010). Several techniques are currently available to evaluate sperm DNA fragmentation, including the TUNEL and Comet assays, which enable direct assessment of DNA fragmentation (Collins *et al.*, 2008; Henkel *et al.*, 2010). SCD and SCSA techniques are also commonly used to measure sperm DNA integrity and involve measuring the vulnerability of chromatin structure to denaturation in order to allow visualization of the sperm nucleus or chromatin compaction status (Henkel *et al.*, 2010; Perez-Cerezales *et al.*, 2012). Collectively, these methods show variability and contradictory results in terms of evaluating sperm nuclear chromatin status, predominantly due to the heterogeneous nature of sperm samples (Parmegiani *et al.*, 2014).

Nevertheless, studies have clearly identified altered protamine ratios in sperm from infertile men with elevated levels of DNA fragmentation (Aoki *et al.*, 2005a; Simon *et al.*,

2010; Castillo *et al.*, 2011; Simon *et al.*, 2011). For example, Aoki *et al.*, (2005a) clearly demonstrated significant association between alterations in P1:P2 ratio in sperm from infertile men with increased levels of DNA fragmentation, poor sperm parameters and poor sperm viability (Aoki *et al.*, 2005b). Fertilisation outcome following the use of such sperm for IVF or ICSI treatments may therefore be at risk of fertilisation failure and/or abnormal embryonic development (Cho *et al.*, 2001; Cho *et al.*, 2003; Depa-Martynow *et al.*, 2012). However, despite the potential ability of an oocyte to repair DNA errors following fertilisation, which is dependent on the extent and type of sperm DNA damage (Sakkas & Alvarez, 2010), concerns still remain. For example, the incorporation of sperm with poor DNA integrity into the oocyte may detrimentally affect the integrity and function of an oocyte, which may lead to abnormal embryonic development, thus risking the health and genetic integrity of the offspring (Raul Fernandez 2008). For example, alterations, insufficiencies, or reduced levels of P1 and P2 content or P1:P2 ratio, including increased DNA fragmentation levels within a sperm, may be detrimental to the fertilised embryo leading to abnormal embryonic development or embryonic death (Cho *et al.*, 2001; Cho *et al.*, 2003; Depa-Martynow *et al.*, 2012). These findings clearly provide evidence to suggest the fundamental requirement of both P1 and P2 in sufficient amounts for successful fertilisation and normal embryonic development.

The mechanism of DNA fragmentation during spermatogenesis and epididymal maturation was described in Chapter 1 (**Figure 1.20**). Abnormalities in protamination and the potential risk of DNA fragmentation incurred during spermatogenesis involve several crucial processes, such as apoptosis, or the defective activity of topoisomerase enzyme II and endonuclease activity (Sakkas & Alvarez, 2010; Francis *et al.*, 2014). Following testicular protamination, sperm undergo further nuclear protamine-DNA compaction via disulfide bridge formation and phosphorylation during epididymal maturation (Oliva, 2006). The

potential for sperm to incur DNA damage during epididymal maturation may detrimentally affect the protamination process, leading to abnormal alterations in protamine ratio. For example, during epididymal maturation, sperm are exposed to biochemical secretion and high levels of ROS (Ollero *et al.,* 2001). Increased levels of ROS may subsequently increase oxidative stress levels and this can cause DNA strands to break in sperm (Cocuzza *et al.*, 2007). Such mechanisms may therefore disrupt the cross-linking of disulfide bonds formed between protamine and sperm nuclear DNA leading to the disassociation of protamine molecules, thus altering the protamine levels in sperm.

While abnormal protamine packaging and DNA fragmentation in sperm are attributed to internal factors during spermatogenesis and post-testicular events, external factors such as chemotherapy, radiotherapy, or environmental toxicants, may also potentially cause increased DNA damage leading to abnormal protamination (Morris, 2002). Currently, IVF and ICSI treatments are widely used to combat infertility disorders among couples. However, it is worrying that the success rate of ART rarely exceed 40% (Yelumalai *et al.,* 2012). This could be attributed to various male/female factors or be related to gametogenesis and chromosomal abnormalities. However, laboratory procedures which preserve and/or manipulate gametes/embryos in an artificial environment may also compromise functional ability (Yelumalai *et al.,* 2012). For example, cryopreservation is a routinely used technique to preserve sperm by sustaining its biological properties for later use (Jain & Paulson, 2006). However, the adverse effects of freezing and thawing upon basic sperm parameters, can influence DNA integrity and crucial sperm proteins which are essential for fertilisation, including PLCζ (Isachenko *et al.*, 2004a; Valcarce *et al.*, 2013; Xiaoli *et al.*, 2013; Paoli *et al.*, 2014). Moreover, mounting evidence demonstrates increased DNA fragmentation levels in sperm following cryopreservation, suggesting the need for sperm DNA damage assessment prior to the use of cryopreserved sperm for ART (Valcarce *et al.,* 2013). While the effect of

cryopreservation upon sperm DNA integrity is clearly evident, there is a high possibility that freezing may also adversely affect protamine content in a sperm thus altering P1:P2 ratios and resulting in poor or failed fertilisation following IVF or ICSI. Although DNA fragmentation assessment is already available for clinical use (Practice Committee of the American Society for Reproductive, 2013), the significance and reliability of such tests in terms of predicting the fertilising ability of a sperm remains contradictory due to variability in the DNA assessment technique used (Parmegiani *et al.*, 2014). However, to date there have been no studies reporting the direct or indirect assessment of protamine levels in cryopreserved sperm in order to evaluate fertilisation ability of a sperm.

To date, investigators have utilised a well characterized direct assessment technique known as acid-urea gel electrophoresis to evaluate protamine content and P1:P2 ratio in nuclear protein extracted from fertile and infertile human sperm (Oliva, 2006). However, several studies have also applied indirect techniques to evaluate protamine content or chromatin structure in sperm from infertile men based upon different fluorochrome or staining methods (Oliva, 2006). For example, chromomycin A3 (CMA3) staining, a competitive binding assay between protamine and CMA3, was used to demonstrate an inverse association between the levels of protamine and CMA3 in sperm (Nasr-Esfahani *et al.*, 2004). Another indirect protamine evaluation was performed using aniline blue dye, which stains histone retention and indicates lower levels of protamine in the mature sperm nucleus (Auger *et al.*, 1990). Moreover, sperm can be treated with sodium dodecyl sulphate (SDS) or dithiothreitol (DTT) to measure the degree of chromatin de-condensation from infertile men as compared to fertile men, since sperm with a lower amount of protamine may also exhibit poorly condensed chromatin (Le Lannou *et al.*, 1986; Jager, 1990). The existing four DNA fragmentation tests described earlier in this chapter are also considered as indirect approaches to measure abnormal protamination in sperm from fertile or infertile men. Since

the use of such indirect methods may depend upon the structure of chromatin, accessibility and DNA integrity of a sperm, the direct quantification of protamine following sperm nuclear extraction and gel electrophoresis are still considered to represent the most reliable of the existing techniques. However the use of such methods for clinical application can be time consuming (Oliva, 2006), and these techniques only provide information on protamine levels and not localisation profile within individual sperm.

Recent reports have described the utilisation of mRNA techniques to determine protamine ratio in infertile men and indicated that protamine mRNA ratio may serve as the best clinical tool to predict male infertility (Rogenhofer *et al.*, 2013). However, working with mRNA is not easy and maintaining consistent approach against mRNA degradation might be difficult in an IVF laboratory. Although these techniques have shown the significance of protamine ratio in relate to male infertility, none have been established for routine clinical utilisation predominantly due to time and cost. Moreover, with the availability of well-established techniques such as basic semen analysis and DNA fragmentation tests, there may not be significant drive to include additional test to evaluate protamine levels in sperm. However, basic semen analysis is known to exhibit high variability (Practice Committee of the American Society for Reproductive, 2013). Since abnormal protamination is not the only cause of DNA fragmentation, it is vital to develop a direct and cost-effective novel biological tool based upon protamine evaluation in sperm from fertile or infertile men, and in cryopreserved sperm, in order to predict sperm integrity and ability to safely fertilise. In addition, such methods may also facilitate the selection of sperm with optimal integrity leading to improved rates of fertilisation. Moreover, existing studies provide data relating to only protamine levels, and do not consider the precise spatial localisation of these proteins in individual sperm. It is therefore unknown as to whether localisation data may be of clinical use.

The general aims of the current chapter were to develop a novel immunofluorescent assay to determine expression levels and localisation profile of P1 and P2 in sperm and then to use this assay to investigate the effect of cryopreservation upon epididymal maturation and protamine levels in the sperm. Sixteen week old mice were used throughout this project, since mice of this age are fully mature with sexual history.

2.1.1 Specific aims of this chapter

1. To test and validate the specificity of monoclonal antibodies raised against P1 and P2 in sperm from mice and boar models. Boar sperm were particularly useful for validation purposes as they only express P1 and not P2. Once validated, antibodies were used to develop a novel immunofluorescent assay to determine the expression levels and spatial localisation pattern of P1 and P2 in sperm from the mouse model.
2. To evaluate and compare the total levels of P1, P2 and the P1:P2 ratio, in sperm collected from three regions of the mouse epididymis (caput, corpus and cauda) in order to investigate whether these parameters change with increasing levels of maturation along the epididymis.
3. To determine and compare DNA fragmentation levels in non-cryopreserved and cryopreserved epididymal mouse sperm (from caput, corpus and caudal epididymal regions) to ascertain whether cryopreservation influences DNA integrity and whether immature sperm are more vulnerable to DNA damage.
4. To specifically evaluate total levels of P1, P2, and the P1:P2 ratio in non-cryopreserved and cryopreserved sperm from the most mature sperm from the mouse cauda epididymis.

2.2 Materials and Methods

2.2.1 Mouse supply and sperm preparation

Epididymides were collected from culled mice (strain: C57BL/6, age: 16 weeks old) obtained from Biomedical Services (BMS), John Radcliffe Hospital, Oxford. In total, nine mice were used for this chapter. Epididymides were dissected into three segments; caput, corpus and cauda (**Figure 2.1**). Each segment was perforated with a 25-gauge hypodermic needle and incubated in 200µl of pre-warmed phosphate buffer saline (PBS) (Dulbecco A, UK) for routine analysis (or in 1ml of cryoprotective solution (CPS) for cryopreservation) at 37°C, 5% carbon dioxide (CO_2) for 30 minutes to allow motile sperm to swim-out (**Figure 2.2**). 30µl of cell suspension was loaded onto a 100µm Leja slide (Standard Count 2 Chambers Slides, Leja, The Netherlands) for Computer Assisted Sperm Analysis (CASA; Software Version 12.3K, Hamilton Thorne Biosciences, Ceros, UK) to determine sperm concentration and velocity distribution. Sperm samples were centrifuged at 800*g* for 3 minutes followed by re-suspension in 200µl of pre-warmed PBS. Sperm pellets were washed twice and the supernatant discarded. Finally, sperm pellets were stored in 200µl PBS.

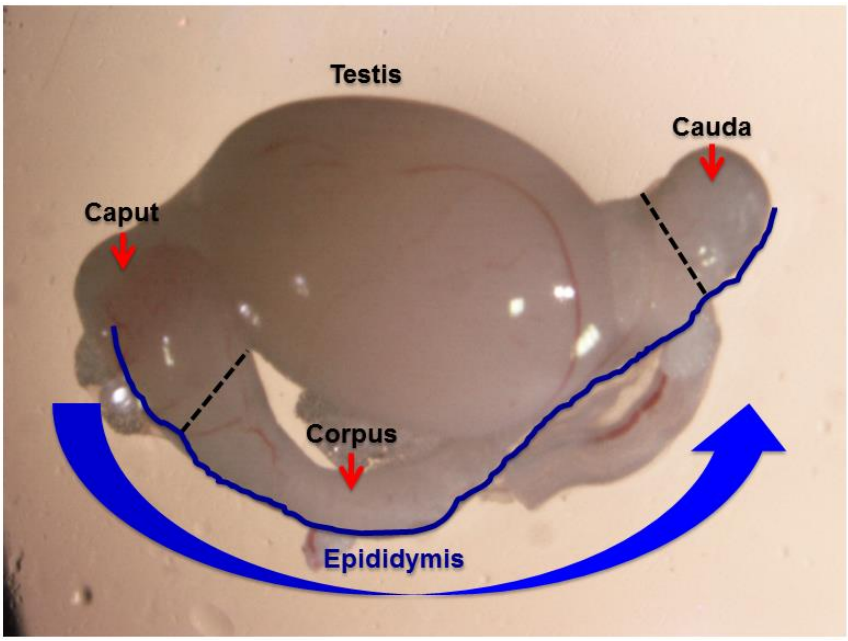


Figure 2.1: Representative image of mouse testis. Blue line represents the epididymis where sperm undergo maturation following post-testicular transport. Red arrows indicate the caput (contains the least mature sperm), corpus and cauda (contains the most mature sperm) Blue arrow indicates the direction of sperm maturation.

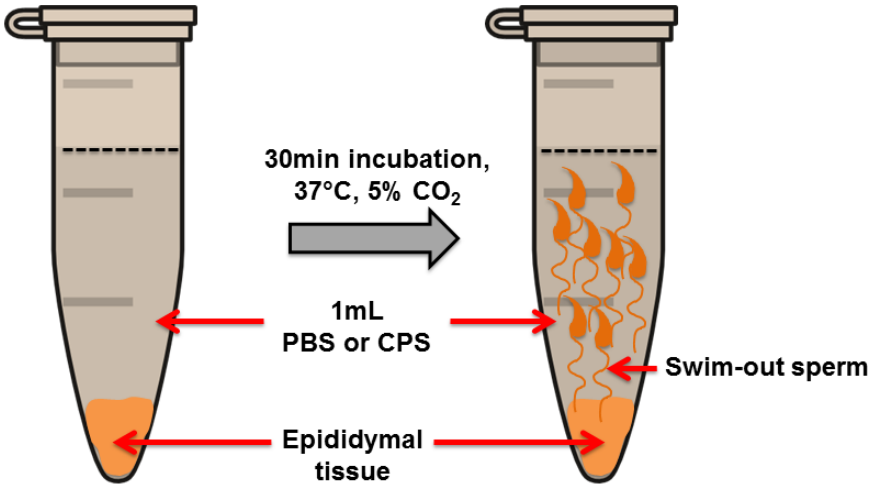


Figure 2.2: Schematic image illustrating the mouse sperm retrieval process from epididymal tissue using the swim-out technique in phosphate buffer saline (PBS) or cryo-protective solution (CPS) for cryopreservation.

2.2.2 Boar semen supply and sperm preparation

Boar semen was obtained from a commercial supplier (JSR Genetics Ltd, Southburn, UK) and delivered to our laboratory in an extender solution at ambient temperature. Upon arrival, boar semen was activated in an incubator set at 35°C for 15 minutes to induce motility. Sperm motility was assessed using a 20µm Leja slide (Standard Count 2 Chambers Slides, Leja, The Netherlands) and Computer Assisted Sperm Analysis (CASA; Software Version 12.3K, Hamilton Thorne Biosciences, Ceros, UK). Sperm were centrifuged at 800*g* for 3 minutes at room temperature and the supernatant containing the extender solution was discarded. Finally, sperm pellets were re-suspended in 200µl of PBS.

2.2.3 Immunofluorescent staining protocol

2.2.3.1 PAP-mould and slide preparation

PAP-moulds were drawn using an Imm-edge pen (Vector Laboratories, Peterborough, UK) on slides pre-coated with 0.01% poly-L-lysine (Sigma Aldrich, UK) (**Figure 2.3**). 100µl of sperm suspension (prepared as described in **Section 2.2.1** and **Section 2.2.2**) was then loaded into PAP-moulds and air-dried for 30 minutes to allow the sample to settle. Sperm samples were washed three times with PBS. Permeabilisation and subsequent immunofluorescence procedures are described in **Sections 2.2.3.2**, **2.2.3.3**, **2.2.3.4** and **2.2.3.8** respectively.

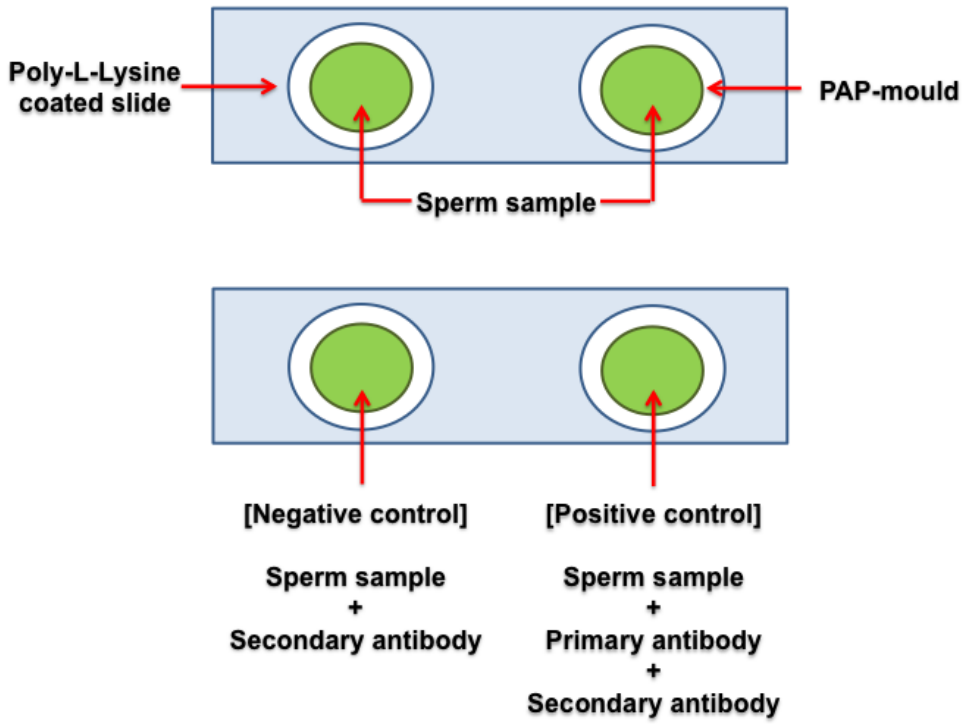


Figure 2.3: Schematic images showing PAP-mould preparation on poly-L-Lysine coated slides. Image also indicates the organization of sperm samples on slides for immunofluorescent staining procedures.

2.2.3.2 Sperm permeabilisation techniques

To quantify the immunofluorescent levels of P1 and P2 in mouse and boar sperm samples were permeabilised in their respective PAP-moulds in order to de-condense the sperm nuclei. Specific permeabilisation buffers were prepared according to the species concerned and are described in **Sections 2.2.3.3** and **2.2.3.4**.

2.2.3.3 Mouse sperm permeabilisation technique

100µl of sperm suspension was loaded into each PAP-mould and air-dried for 30 minutes to allow sperm to settle prior to permeabilisation. Excessive sperm suspension was aspirated from the PAP-moulds and the slide was washed three times with PBS. Sperm samples were then permeabilised as described by Zao *et al*. (2004) with minor modifications. 100µl of solution containing 6mM DTT (Sigma Aldrich, UK) + 50mM Tris HCL (Sigma

Aldrich, UK) was first added onto slides for one hour. Slides were washed twice with PBS and the samples further de-condensed and blocked with a buffer containing 0.5% Triton X-100 (Sigma Aldrich, UK) + 3% Bovine Serum Albumin (BSA) (Sigma Aldrich, UK) for 3 hours. Following permeabilisation, sperm samples were fixed with 100µl of 4% paraformaldehyde (PFA) for 10 minutes. Finally, slides were washed twice with PBS prior to the immunocytochemistry procedure (Zhao *et al.*, 2004).

2.2.3.4 Boar sperm permeabilisation technique

Sperm samples were aspirated from PAP-moulds and slides were washed three times with PBS. Sperm samples were permeabilised as described by Heijden *et al*. (2006) with minor modifications (van der Heijden *et al.*, 2006) with 100µl of solution containing 25mM DTT + 200IU Heparin + 0.2% Triton X-100 for 30 minutes. Samples were further permeabilised and blocked with 0.5% Triton X-100 (Sigma Aldrich, UK) + 3% BSA (Sigma Aldrich, UK) for 3 hours and fixed with 100µl of 4% PFA for 10 minutes. Finally, slides were washed twice with PBS prior to the immunocytochemistry procedure.

2.2.3.5 Validation of P1 and P2 antibodies

Anti-protamine 1 (Hup1N) and anti-protamine 2 (Hup2B) monoclonal antibodies (raised in mouse using highly purified protamine from pool of human sperm) were purchased from Briar-Patch (LLC, USA). Prior to protamine immunofluorescent quantification analysis in mouse sperm, the concentration and specificity of the Hup1N and Hup2B antibodies were validated as described in the following sections.

2.2.3.6 Optimisation of P1 and P2 antibody concentration

The different concentrations of Hup1N, Hup2B (Briar-Patch, LLC, USA) and secondary fluorescent antibody (Alexa Fluor 488 F [ab']$_2$ fragment rabbit anti-mouse IgG; Invitrogen) used for optimisation experiments are shown in **Table 2.1** and **Table 2.2**. The first sets of experiments were performed to optimise the primary antibody concentration ranging from 5µg/ml to 25µg/ml while maintaining the secondary antibody at a constant concentration of 10µg/ml (**Table 2.1**).

Table 2.1: Different concentrations of primary monoclonal protamine antibodies Hup1N and Hup2B used for optimisation.

Hup1N/Hup2B antibody concentration	Secondary antibody concentration
5µg/ml	**10µg/ml**
10µg/ml	**10µg/ml**
20µg/ml	**10µg/ml**
25µg/ml	**10µg/ml**

Table 2.2: Different concentrations of secondary antibody used for optimisation using an optimum concentration of primary antibody (20µg/ml) as determined earlier.

Anti-P1 and P2 antibody concentration	Secondary antibody concentration
20µg/ml	**2µg/ml**
20µg/ml	**4µg/ml**
20µg/ml	**8µg/ml**
20µg/ml	**10µg/ml**

Subsequently, the next set of experiments to optimise secondary antibody concentration were performed by maintaining the primary antibody concentration at a constant level of 20µg/ml, the optimum concentration of primary antibody determined from the first set of experiments, with secondary antibody concentration ranging from 2µg/ml to 10µg/ml as shown in **Table 2.2**.

2.2.3.7 Specificity testing of P1 and P2 antibodies

Following the optimisation of primary and secondary antibody concentration, the specificity of each anti-P1 and anti-P2 antibody was determined using an excess amount of P1 and P2 immunogenic peptide at a concentration of 80µg/ml, in both non-cryopreserved and cryopreserved mouse sperm. Since boar sperm do not express P2, the anti-P2 antibody should therefore not react with boar sperm. Therefore, immunoreactivity of anti-protamine antibodies in boar sperm was performed to further determine the specificity of these antibodies, and whether P1 and P2 could be detected in a distinct manner.

2.2.3.8 Immunofluorescent staining of P1 and P2 in mouse sperm

Immunofluorescence analysis was used to evaluate total levels of P1 and P2 in non-cryopreserved and cryopreserved mouse sperm prepared earlier by swim-out technique. Following permeabilisation (**Section 2.2.3.2**), samples were incubated overnight at 4°C with 20µg/ml Hup1N and 20µg/ml Hup2B monoclonal antibodies (Briar Patch Bioscience, LLC, USA) in PBS/0.05% BSA on separate PAP-moulds. Negative controls were incubated in the absence of primary antibody. Subsequently, samples were washed in PBS and incubated at room temperature for 1 hour with 10µg/ml of secondary fluorescent antibody (Alexa Fluor 488 F [ab']$_2$ fragment rabbit anti-mouse IgG; Invitrogen, UK).

Finally, slides were washed thrice with PBS and mounted with 5µl Vectashield H-1200 mountant for fluorescence containing 4'-6'-diamidino-2-phenylindole (DAPI) (Vector, UK), and a 20mm x 20mm glass cover slip. P1 and P2 immunofluorescence distribution and localisation were identified using a fluorescence microscope (80*i*, Nikon) and a fluorescein isothicyanate filter (FITC; wavelength 488nm) for green P1 and P2 fluorescence using an exposure time of 400ms. Slides were imaged at x40 magnification. Additional representative confocal images were also captured at 100x magnification using an Olympus Fluoview FV1000 inverted IX81 confocal laser microscope (Department of Pathology, University of Oxford). Images were acquired at wavelengths of 488nm for green and differential interference contrast (DIC) channels were used for bright field imaging.

2.2.4 Cryopreservation of mouse sperm

2.2.4.1 Preparation of cryoprotective solution (CPS)

Cryoprotective solution (CPS) was prepared according to protocols described by Yildiz *et al*. (2007) by dissolving 18% of Raffinose (Sigma Aldrich, UK) and 3.0% of dried skimmed milk in water and pre-warmed in a 60°C water bath. The solution was mixed gently by inversion and centrifuged at 14,000*g* for 10 minutes. Pellets were discarded and the supernatant filtered using a sterile 0.45µm filter. Filtered CPS was stored at -20°C. Prior to cryopreservation, the CPS solution was pre-warmed in 37°C water bath for 10 minutes and mixed with 0.1M of fructose (Sigma Aldrich, UK).

2.2.4.2 Sperm cryopreservation protocol

Mouse caudal sperm retrieved via swim-out as described in **Section 2.2.1** were cryopreserved in liquid nitrogen (LN_2) according to protocols previously reported by Nakagata *et al*. (2000) and Yildiz *et al*. (2007). A syringe aspirator was prepared prior to cryopreservation. A cryopreservation-straw tip (Cryo-Bio System, France) was first inserted into the end of a cryopreservation-straw (Cryo-Bio System, France) (**Figure 2.4A**). Next, a 1ml syringe (BD Medical, USA) nozzle was fitted with a pipette tip, followed by a cryopreservation-straw (cotton end) into the pipette tip (**Figure 2.4B**). Approximately 500µl of sperm suspension was cautiously aspirated into the cryopreservation-straw via the syringe aspirator and avoiding bubble formation. The cryopreservation-straw was then removed from the syringe aspirator and sealed using a plastic film sealer (PFS). Samples were frozen slowly in LN_2 vapour for 30 minutes. Finally, cryopreservation-straws were stored in LN_2 until required. The remaining 1ml of sperm suspension was washed twice by centrifugation at 800*g* for 3 minutes and re-suspended in 200µl of pre-warmed PBS.

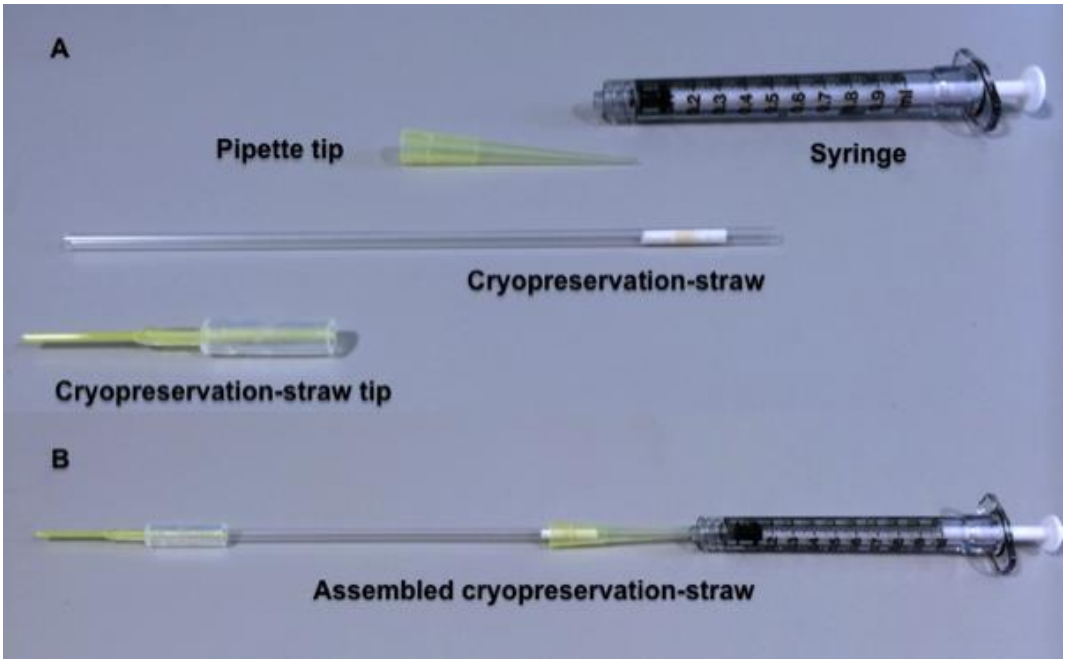


Figure 2.4: Representative image showing preparation of the cryopreservation straw syringe aspirator for cryopreserved sperm suspension. (**A**) Unassembled cryopreservation straw and (**B**) complete assembled cryopreservation straw.

2.2.4.3 Sperm thawing protocol

Cryo-straws were removed from the LN_2 tank and immediately thawed in a 37°C water bath for 1 minute. Thawed samples were dispensed from cryopreservation-straws into 1.5ml tubes and incubated in a 37°C water bath for 15 minutes. Samples were then washed by centrifugation to remove CPS and re-suspended with warm PBS. Basic sperm parameters were assessed using CASA. Following another washing step with centrifugation at 800*g* for 3 minutes, the supernatants were discarded. Finally, pellets were re-suspended and stored in 200µl of warm PBS (Nakagata, 2000; Yildiz *et al.*, 2007).

2.2.5 DNA fragmentation assessment in mouse sperm

DNA integrity of epididymal mouse sperm was evaluated using a commercially available DNA fragmentation kit (*Mus-halomax* kit; Halotech DNA, SL Spain). A tube containing Agarose Cell Support (ACS) was first incubated in a 95°C-100°C water bath for 5 minutes to allow the agarose to liquefy and then equilibrated at 37°C for a further 5 minutes. Approximately 25µl of non-cryopreserved and thawed mouse sperm suspension from each epididymal region previously stored in PBS was mixed gently with 50µl of liquefied agarose and maintained at 37°C. Subsequently, 1.5µl -2.0µl drops of sperm suspension was placed onto marked wells on glass slides and covered with a cover slip avoiding air bubble formation. Slides were then placed horizontally on top of a cold surface for 5 minutes to allow the agarose to solidify. The cover slips were carefully removed and slides immersed in a base lysis solution (BLS) for 5 minutes, then drained and washed with distilled water. Next, slides were dehydrated in 70% ethanol for 2 minutes then drained and washed with distilled water. Slides were then incubated in 100% ethanol for 2 minutes, drained and allowed to dry. Slides were finally mounted with 1.0µl of DAPI (Vector, UK), and covered with 20mm x 20mm glass cover slips for visualization and imaging.

2.2.6 Sperm selection and the measurement and quantification of DNA halos in mouse sperm

DNA fragmentation levels in mouse sperm were assessed based upon the size of the sperm head and resultant DNA halo using ImageJ software (US National Institute of Health). Prior to analysis, the set scale tool in ImageJ software was used to set the pixel value corresponding to the size of a mouse sperm head (**Figure 2.5**). Subsequently, the outline of sperm heads and DNA halos were measured and quantified in micrometres (µm) (**Figure 2.6**). The sperm head (as indicated by the yellow dotted circle) and DNA halos (as indicated by the red dotted circle) show the specific area measured (**Figure 2.7**).

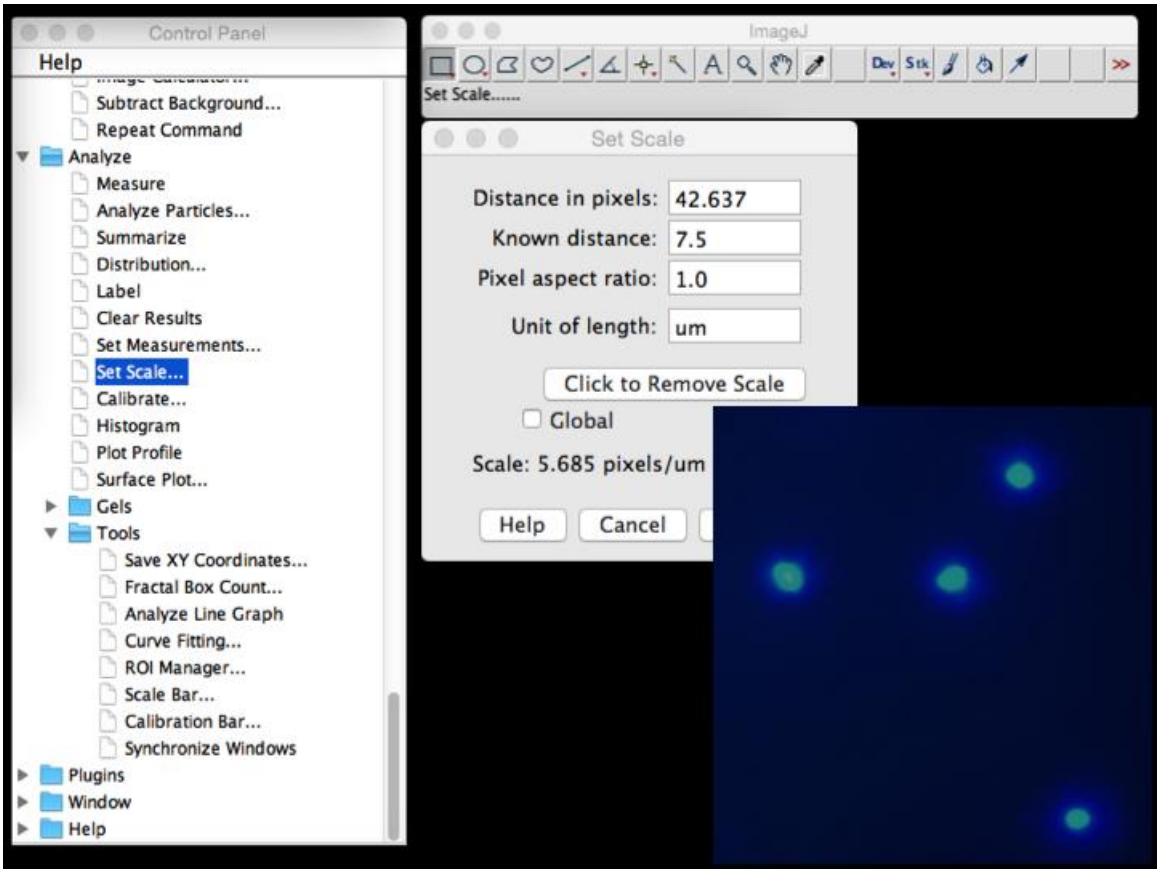


Figure 2.5: Screen capture of the Region of Interest (ROI) manager tool from ImageJ software used to set the scale for area measurement of DNA halos based upon the approximate size of the mouse sperm head (7.5µm).

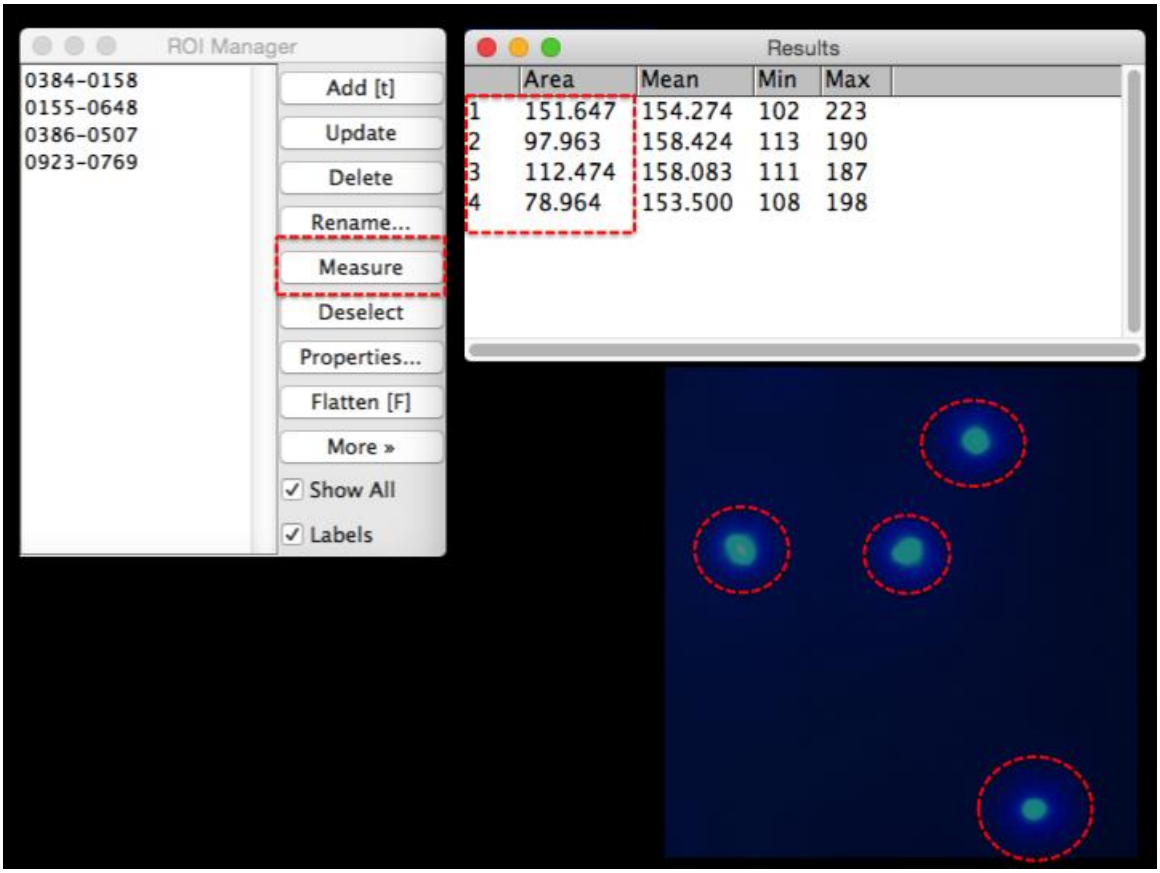


Figure 2.6: Screen capture of the Region of Interest (ROI) manager tool from ImageJ software used to outline DNA halos as indicated by red dotted circles. The outlined DNA halos were measured and the area value for each sperm was quantified as indicated by the red dotted square.

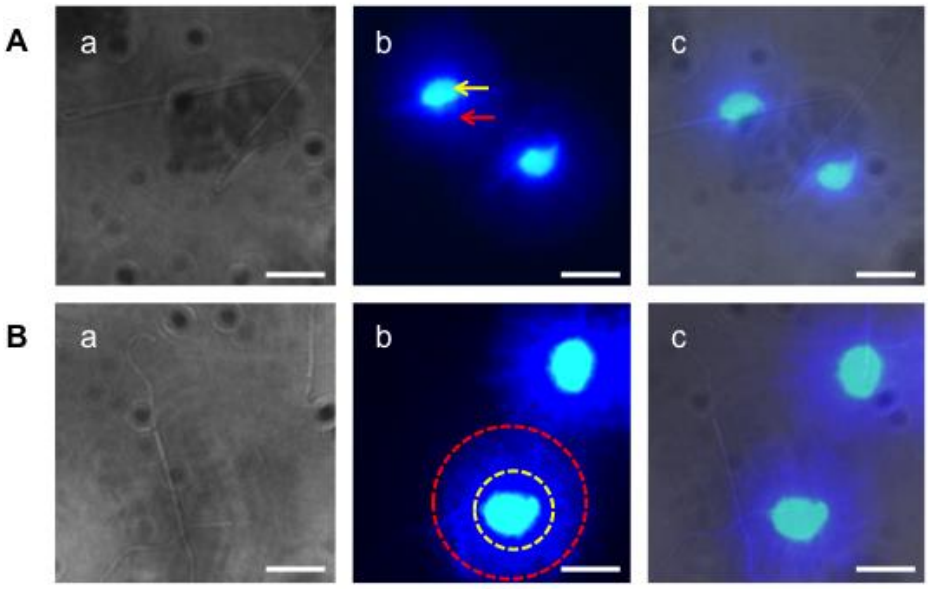


Figure 2.7: Representative microscopic images of (a) bright-field (b) nuclei (DNA fragmentation) and (c) overlay of caudal sperm from a 16 week old mouse (**A**) prior to cryopreservation and (**B**) following cryopreservation. Yellow arrow - sperm head, red arrow - DNA halos around the sperm head prior to cryopreservation. Dotted circles; yellow- enlarged sperm head and red- enlarged DNA halos following cryopreservation. Image was captured at x40 magnification. White scale bar indicates 7.5µm for sperm head in **Figure 2.7A**. Scale bar for expanded sperm head (yellow circle) and DNA halos (red circle) in **Figure 2.7B** indicates 107.0µm and 395.0µm respectively.

2.2.7 Sperm selection, quantification and image analysis to evaluate the total fluorescence levels of P1 and P2 in mouse sperm

Bright field (BF) images were captured (40X magnification) and used to select sperm heads for analysis. Only sperm where the head was still attached to the tail were chosen for analysis, ensuring that such sperm did not overlap with others. Sperm heads were outlined using the region of interest manager (ROI) and quantification tool from ImageJ software (US National Institute of Health) (**Figure 2.8**). Outlined sperm heads from BF images were overlaid onto corresponding fluorescence images (**Figure 2.9**) and the total number of sperm selected was combined to determine mean relative fluorescence. Approximately 100 cells were analysed for each sample. A mean value for fluorescence intensity was calculated for every field of view.

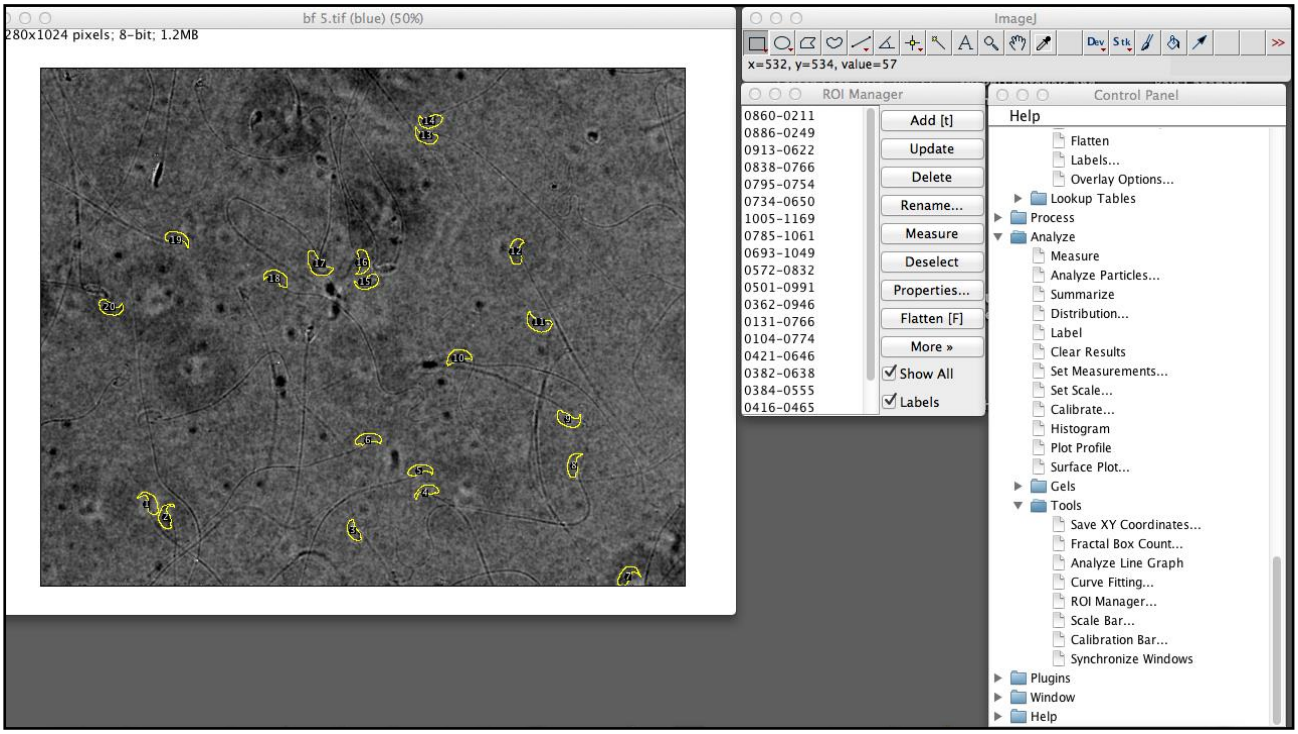


Figure 2.8: Screen capture of the Region of Interest (ROI) manager tool from ImageJ software used to outline each sperm in bright field images.

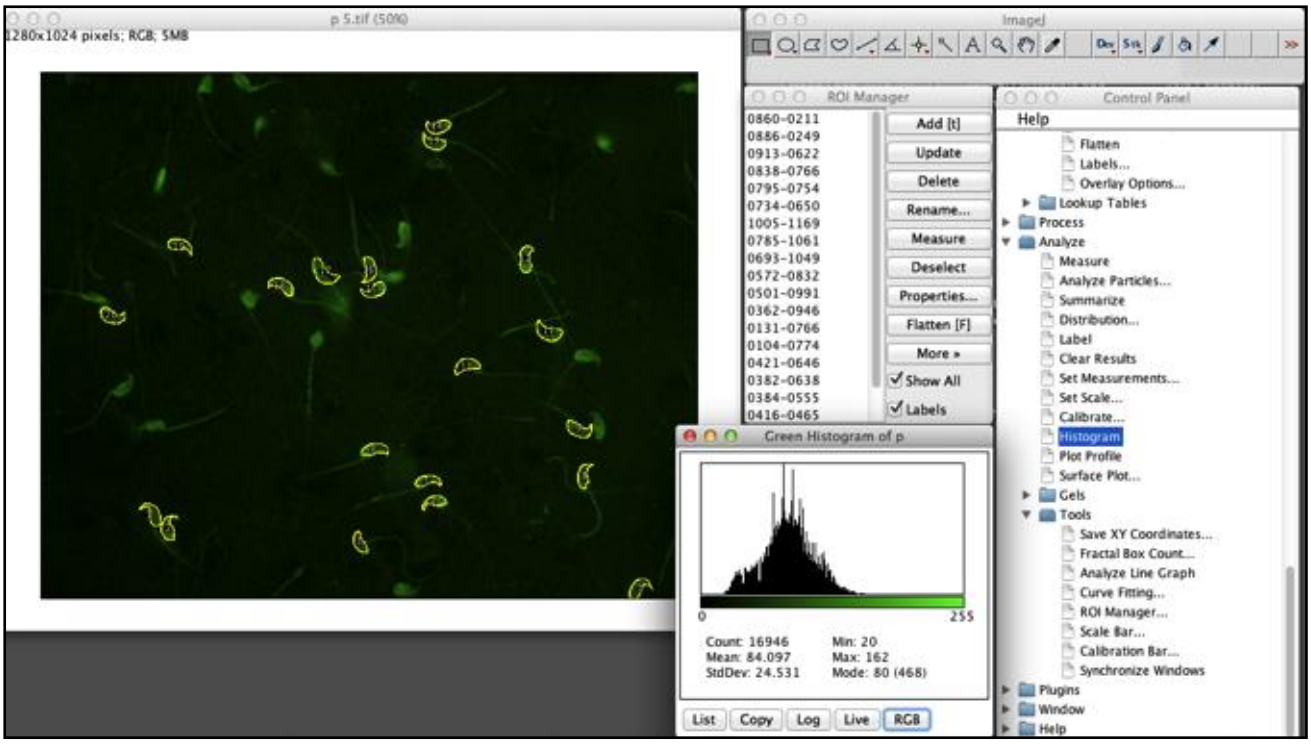


Figure 2.9: Screen capture of the Region of Interest (ROI) manager tool from ImageJ software used to overlay each sperm outlined in bright field onto a green fluorescence images. Subsequently, the total levels of green fluorescence intensity were measured using the colour histogram tool.

2.2.8 Statistical analysis

Data obtained were analysed using GraphPad Prism Software (Version 5, USA) and IBM SPSS 20.0 for Windows (IBM corp.; Chicago, Illinois). All data were first analysed using the Shapiro-Wilks test to confirm normal distribution across numerical values. Secondly, variance homogeneity of data (homoscedasticity) was also assessed using the Levene's test. When data (x) did not match parametric assumptions, they were transformed using log(x), square root ($\sqrt{x}$) and arcsine square root (arcsin $\sqrt{x}$) methods and the most suitable transformation was chosen.

The effect of epididymal region (caput, corpus and cauda) on sperm concentration, total motility, progressive motility, P1 and P2 levels, and P1:P2 ratio was evaluated by One-Way Analysis of Variance (ANOVA), followed by a Bonferroni post-hoc test for pairwise comparisons. Each mouse (n=3) was considered as an independent statistical case.

In order to evaluate the effect of cryopreservation, each mouse (n=5) was treated as an independent statistical case and an unpaired *t*-test was carried out to compare cryopreserved with non-cryopreserved (fresh) sperm. The parameters evaluated included sperm concentration, total motility, progressive motility, P1 and P2 levels, and P1:P2 ratio.

Finally, the effects of cryopreservation and epididymal region on chromatin fragmentation (head size and halo size) were evaluated by taking each sperm as an independent statistical case. Subsequently, a two-way ANOVA followed by a Bonferroni post-hoc test for pairwise comparisons was carried out. Factors included: a) epididymal region and b) cryopreserved vs. non-cryopreserved sperm. A P-value ≤ 0.05 was considered to be statistically significant.

2.3 Results

2.3.1 Anti-protamine antibodies and optimisation of secondary antibody concentration

Prior to the specificity validation of the protamine monoclonal antibodies (Hup1N and Hup2B) used in this chapter, the concentration of these antibodies, and their corresponding secondary antibody, were first determined using mouse caudal sperm. Several studies were also used as a reference to optimise the concentration of these antibodies (Zhao *et al.*, 2004; van der Heijden *et al.*, 2006). The primary monoclonal protamine antibodies were first optimised as described in **Section 2.2.3.6** and **Table 2.1**.

The effects of different Hup1N and Hup2B antibody concentrations are shown in **Figure 2.10**. Green fluorescence intensity increased with increasing concentrations of Hup1N (**Figure 2.10A-D)** and Hup2B **(Figure 2.11A-D**) antibodies ranging from 5µg/ml to 25µg/ml with the secondary antibody concentration maintained at 10µg/ml. As indicated by the red

circle, 20µg/ml of Hup1N and Hup2B antibodies was selected as the optimised concentration since other concentrations appeared to show a punctuated form of protamine localized across the sperm head. The highest concentration (25µg/ml) of protamine antibody was considered to be excessive with markedly high fluorescence intensity.

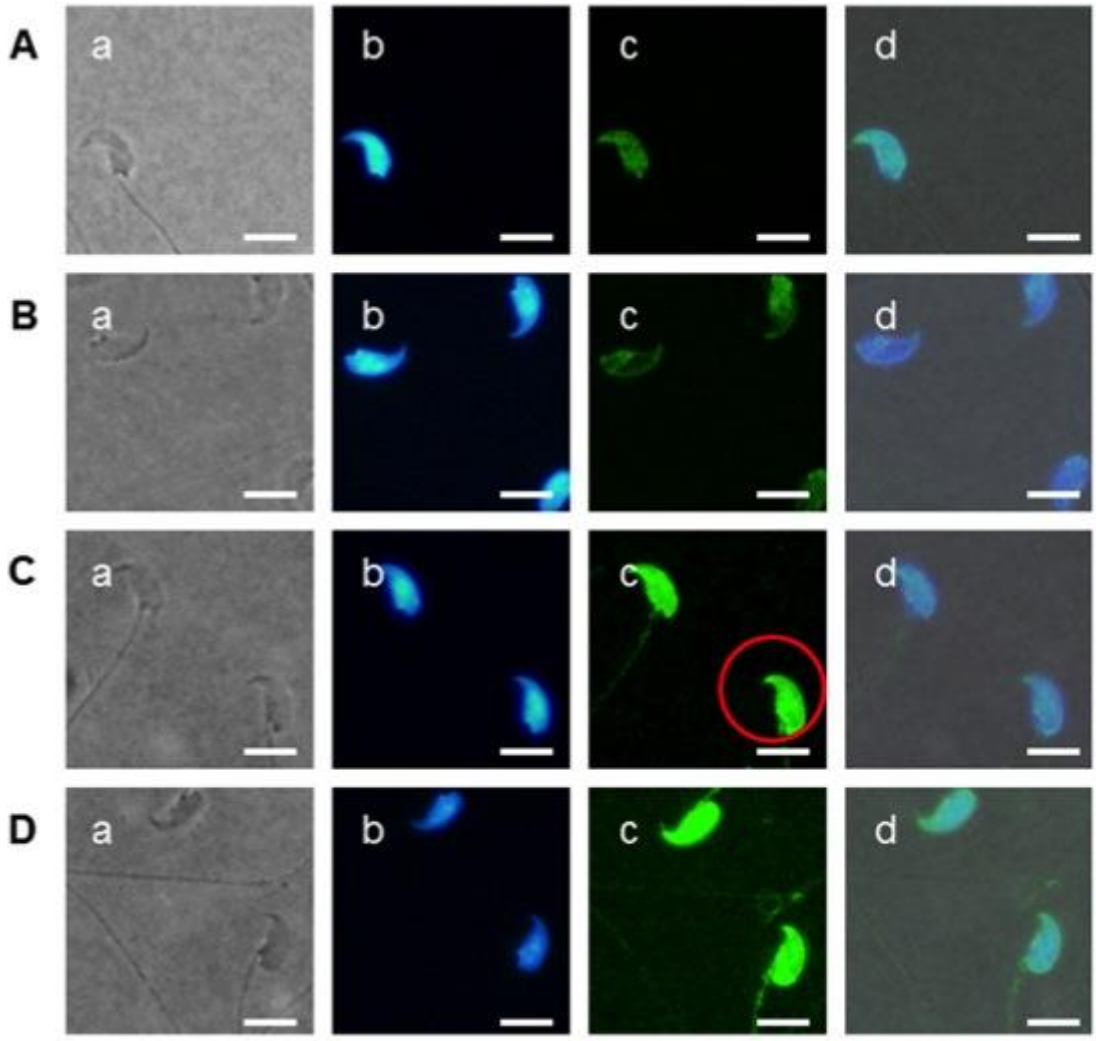


Figure 2.10: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 1 (P1), and (d) overlay of mouse caudal sperm for optimisation of primary antibody concentration. The secondary antibody concentration was maintained at 10µg/ml. Figure shows the presence of P1 with different anti-protamine 1 antibody (Hup1N) concentrations at (**A**) 5µg/ml (**B**) 10µg/ml (**C**) 20µg/ml and (**D**) 25µg/ml. Red circle indicates the presence of P1 with the optimised primary antibody concentration. Image was captured at x40 magnification. White scale bar indicates 7.5µm.

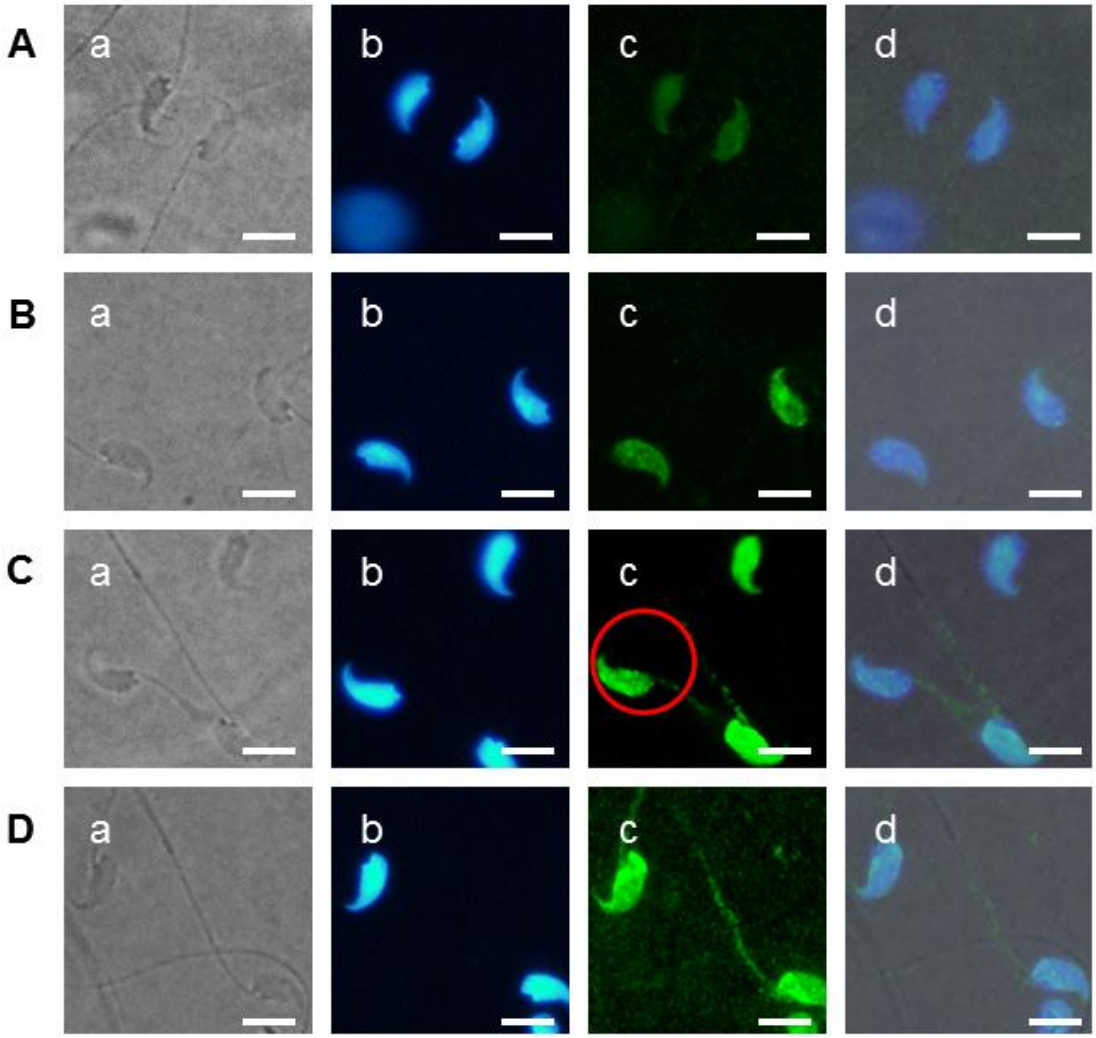


Figure 2.11: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 2 (P2), and (d) overlay of mouse caudal sperm for optimisation of primary antibody concentration. The secondary antibody concentration was maintained at 10µg/ml. Figure shows the presence of P2 with different anti-protamine 2 antibody (Hup2B) concentrations at (**A**) 5µg/ml (**B**) 10µg/ml (**C**) 20µg/ml and (**D**) 25µg/ml. Red circle indicates the presence of P2 with the optimised primary antibody concentration. Image was captured at x40 magnification. White scale bar indicates 7.5µm.

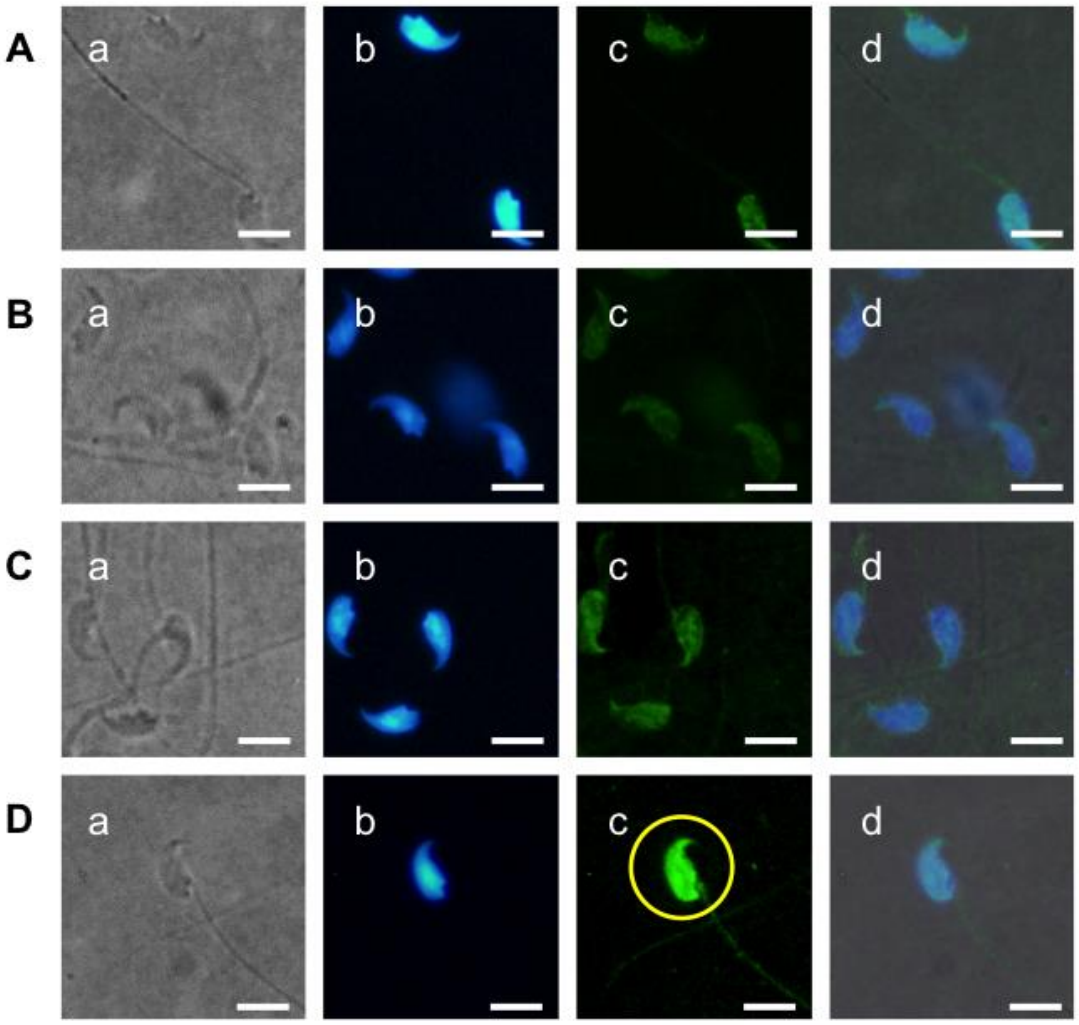


Figure 2.12: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 1 (P1), and (d) overlay of mouse caudal sperm for optimisation of secondary antibody concentration. Anti-protamine 1 antibody (Hup1N) concentration was maintained at 20µg/ml. Figure shows the presence of P1 of different secondary antibody concentration at (**A**) 2µg/ml (**B**) 4µg/ml (**C**) 8µg/ml and (**D**) 10µg/ml. Yellow circle indicates the presence of P1 with the optimised primary antibody concentration. Image was captured at x40 magnification. White scale bar indicates 7.5µm.

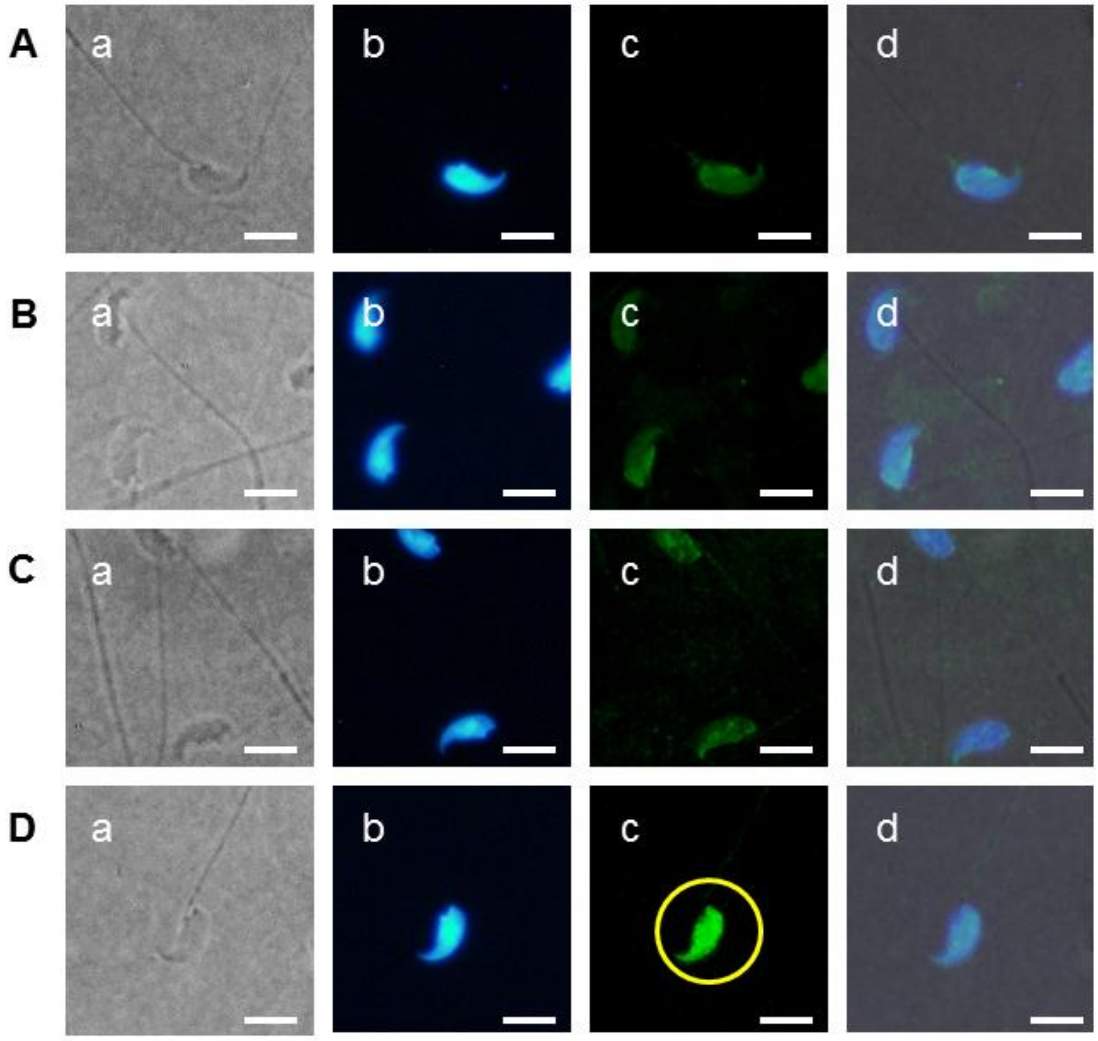


Figure 2.13: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 2 (P2), and (d) overlay of mouse caudal sperm for secondary antibody concentration optimisation. Anti-protamine 2 antibody (Hup1N) concentration was maintained at 20µg/ml. Figure shows the presence of P2 of different secondary antibody concentration at (**A**) 2µg/ml (**B**) 4µg/ml (**C**) 8µg/ml and (**D**) 10µg/ml. Yellow circle indicates the presence of P2 with the optimised primary antibody concentration. Image was captured at x40 magnification. White scale bar indicates 7.5µm.

The various secondary antibody concentrations used for optimisation are shown in **Figure 2.12** and **Figure 2.13**. Green fluorescence intensity increased with increasing concentrations of secondary antibody ranging from 2µg/ml to 10µg/ml (**Figure 2.12A-D**) and **(Figure 2.13A-D**), while Hup1N and Hup2B were maintained at an optimised concentration of 20µg/ml. As indicated by the yellow circle, 10µg/ml of secondary antibody was selected as the optimised concentration for both Hup1N and Hup2B, since other concentrations of

secondary antibody used showed much reduced fluorescence intensity with punctuated localisation of protamine across the sperm head.

2.3.2 Protamine antibody specificity testing in boar and mouse sperm by peptide blocking

Specificity of the two monoclonal antibodies raised against P1 (Hup1N) and P2 (Hup2B), which are known to be immunoreactive against P1 and P2 in mouse, boar, and various mammalian species, was assured by the manufacturer and supported by numerous publications (Stanker *et al*., 1987; Stanker *et al*., 1993). Protamine is known to be a species-specific protein and boar sperm express only P1 and not P2. Therefore, boar sperm were stained with both Hup1N (**Figure 2.14A**) and Hup2B (**Figure 2.15**) to demonstrate the presence of P1 and absence of P2 respectively. In addition, boar sperm were treated with an excessive amount of the corresponding P1 (**Figure 2.14B**) peptide to block the specific binding of Hup1N in order to further validate the specificity of the monoclonal antibodies used.

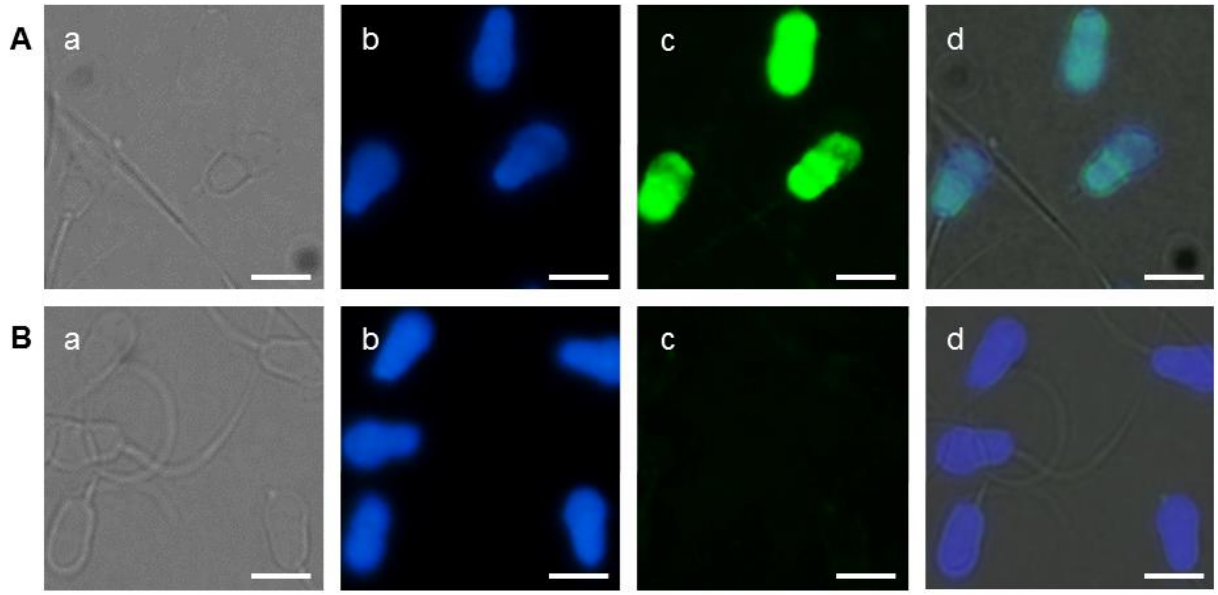


Figure 2.14: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 1 (P1), and (d) overlay of boar sperm following peptide blocking. Figure (**A**) showing the presence of P1 in boar sperm prior to immunogenic peptide treatment and (**B**) showing the absence of P1 in boar sperm following immunogenic peptide treatment. Image was captured at x40 magnification. White scale bar indicates 9.0µm.

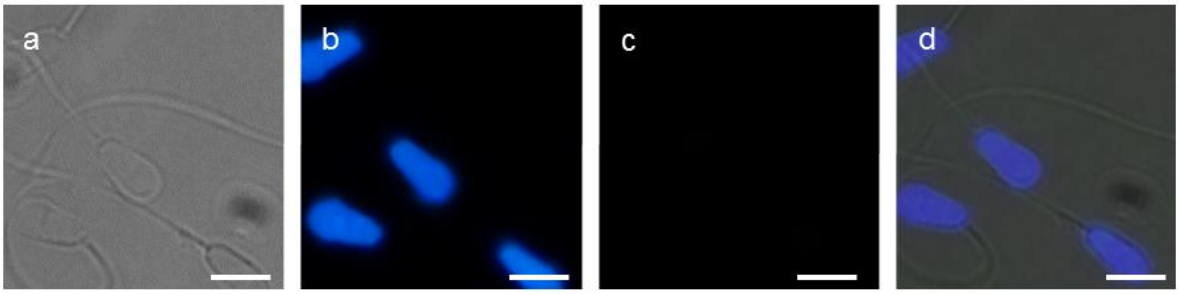


Figure 2.15: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 2 (P2), and (d) overlay of boar sperm showing the absence of P2. Image was captured at x40 magnification. White scale bar indicates 9.0µm.

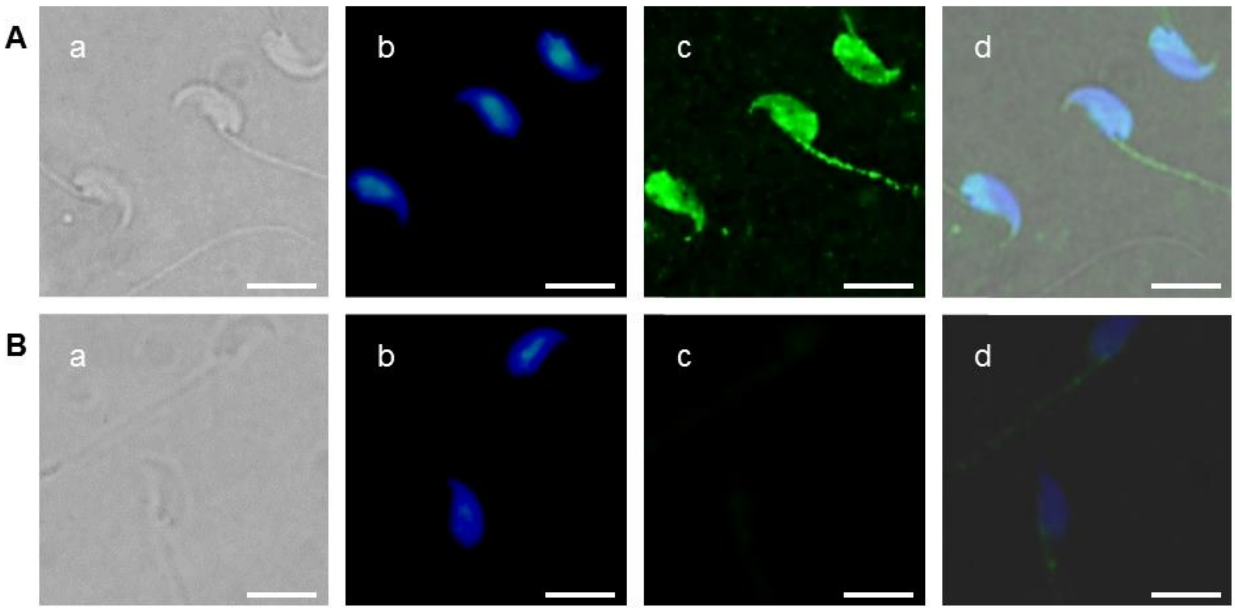


Figure 2.16: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 1 (P1), and (d) overlay of mouse caudal sperm following peptide blocking. Figure (**A**) showing the presence of P1 in mouse sperm prior to immunogenic peptide treatment and (**B**) showing the absence of P1 in mouse sperm following immunogenic peptide treatment. Image was captured at x40 magnification. White scale bar indicates 7.5µm.

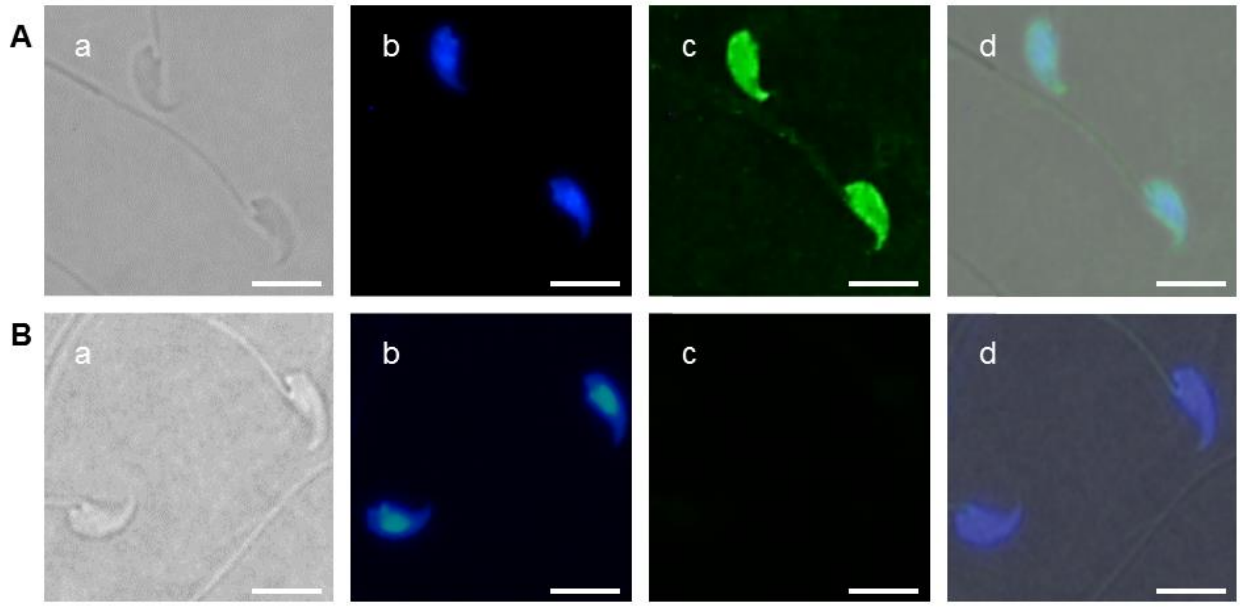
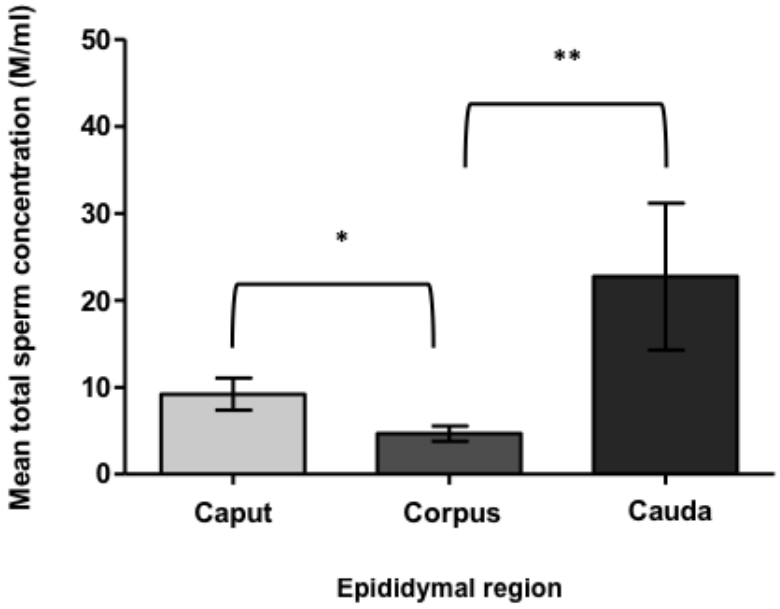


Figure 2.17: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 2 (P2), and (d) overlay of mouse caudal sperm following peptide blocking. Figure (**A**) showing the presence of P2 in mouse sperm prior to immunogenic peptide treatment and (**B**) showing the absence of P2 in mouse sperm following immunogenic peptide treatment. Image was captured at x40 magnification. White scale bar indicates 7.5µm.

Furthermore, specificity of the protamine antibodies was further determined in mouse caudal sperm by treating with excessive amounts of the corresponding immunogenic Hup1N and Hup2B peptides to block the specific binding of P1 and P2. The specific binding of P1 (**Figure 2.16A**) and P2 (**Figure 2.17A**) were successfully blocked following immunogenic peptide treatment, as evidenced by the absence of P1 (**Figure 2.16B**) and P2 (**Figure 2.17B**), which further confirmed specificity of the antibodies used.

2.3.3 Total levels of P1 and P2 in sperm retrieved from mouse epididymides

Total levels of P1 and P2 were evaluated in sperm retrieved from the epididymides of 16 week old mice (n = 3). Prior to analysis, basic sperm parameters such as concentration, motility, and progressive motility were determined by CASA. In general, sperm concentration (**Figure 2.18, Anova table 2.1**), motility and sperm progressive motility (**Figure 2.19**) increased significantly ($P \leq 0.05$; as indicated by One-Way ANOVA) from caput, corpus to cauda epididymides.



Anova table 2.1					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	530.107	2	265.053	3.516	0.098
Within Groups	452.293	6	75.382		
Total	982.400	8			

Figure 2.18: Mean total sperm concentration in epididymal sperm from 16 week old mice (n=3) using Computer Assisted Sperm Analysis (CASA). Total sperm concentration is expressed as millions of sperm per millilitre (M/ml). Asterisks (***, ****) denote statistically significant differences ($P \leq 0.05$) between caput and corpus, corpus and cauda respectively. Data are show as mean ± SEM.

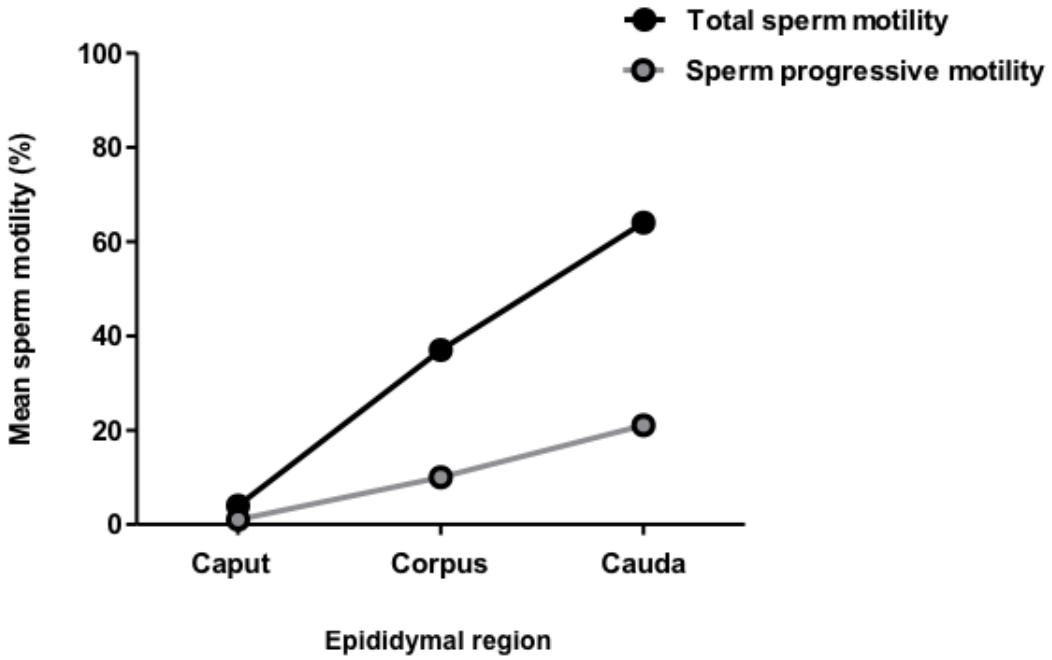


Figure 2.19: Mean sperm motility of epididymal sperm from 16 week old mice (n=3) using Computer Assisted Sperm Analysis (CASA). Total sperm motility and sperm progressive motility is expressed as percentages. Data are shown as mean percentages.

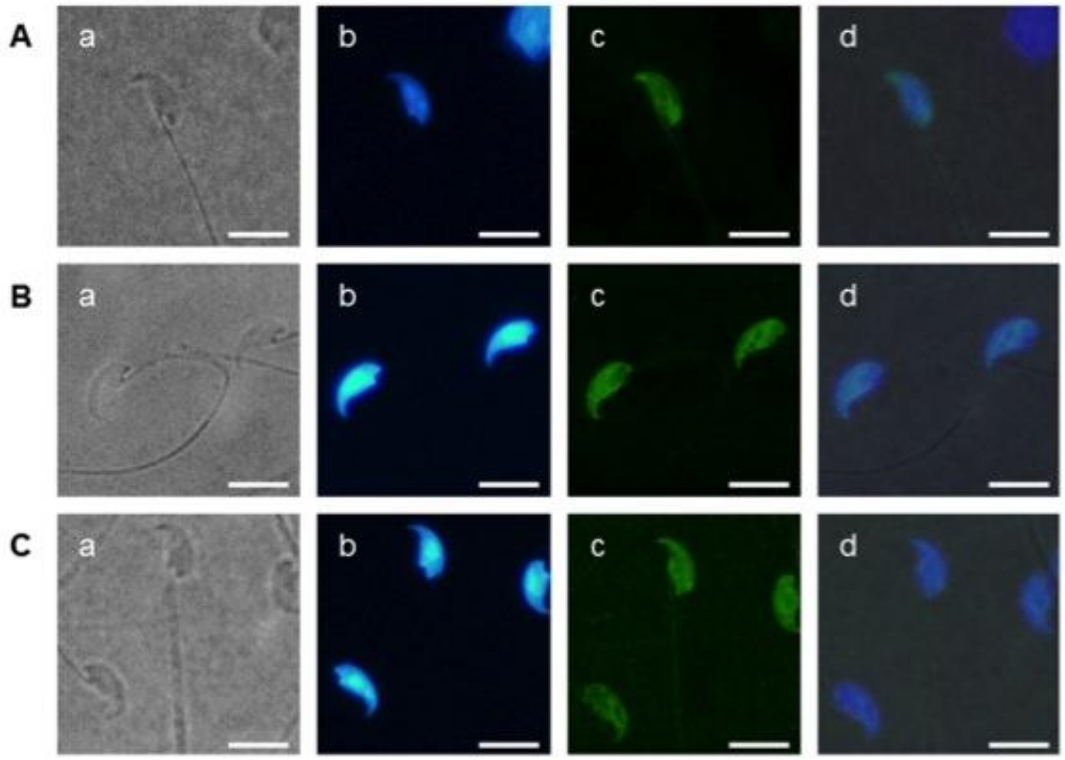


Figure 2.20: Representative microscopic images of (a) bright-field (b) nuclei, (c) protamine 1 (P1), and (d) overlay of epididymal mouse sperm. Figure shows the presence of P1 in (**A**) caput, (**B**) corpus and (**C**) caudal sperm. Image was captured at x40 magnification. White scale bar indicates 7.5µm.

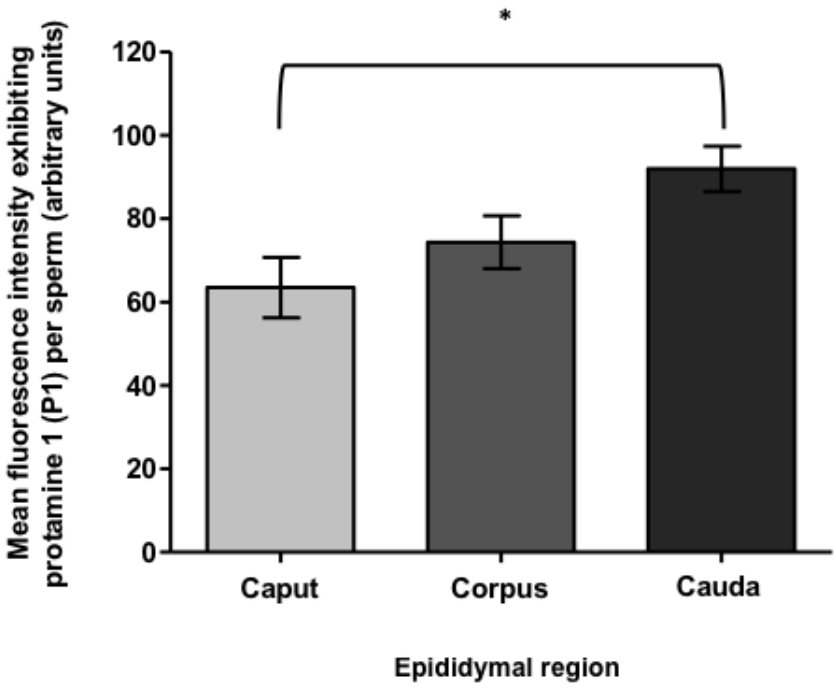


Figure 2.21: Mean relative protamine 1 (P1) fluorescence levels in sperm from caput, corpus, caudal epididymal regions of 16 week old mice (n=3). Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisks (*) denote statistical significant differences ($P \leq 0.05$) in the total levels of P1 between caput and cauda epididymal sperm. Data are shown as mean ± SEM.

Total levels of P1 and P2 in fresh mouse sperm were analysed in sperm retrieved from each epididymal region (caput, corpus, cauda) to evaluate the overall trend of P1 and P2 in association with epididymal maturation. The presence and localisation of P1 expressed across the sperm head is shown in **Figure 2.20**. Total levels of P1 in the total number of sperm (n=300) from each epididymal region (caput, corpus and cauda) were evaluated and the mean fluorescence levels of P1 were determined as 63.5 ± 7.2AU, 74.3 ± 6.3AU and 91.9 ± 5.5AU respectively. As demonstrated in **Figure 2.21**, the mean total levels of P1 increased gradually as sperm matured through the epididymal region. Total levels of P1 in cauda sperm were significantly higher than that in the caput $P \leq 0.05$; as indicated by One-Way ANOVA).

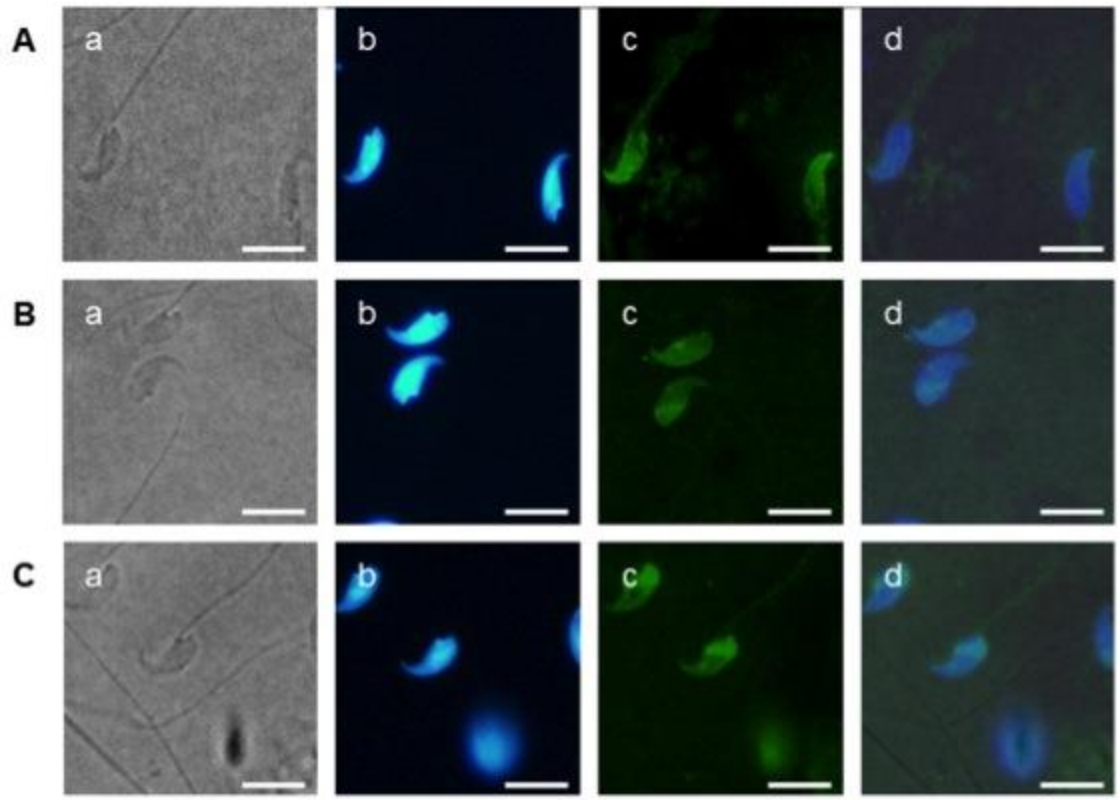


Figure 2.22: Microscopic images of (a) bright-field (b) nuclei, (c) protamine 2 (P2), and (d) overlay of epididymal mouse sperm. Figure shows the presence of P2 in (**A**) caput, (**B**) corpus and (**C**) caudal sperm. Image captured at x40 magnification. White scale bar indicates 7.5µm.

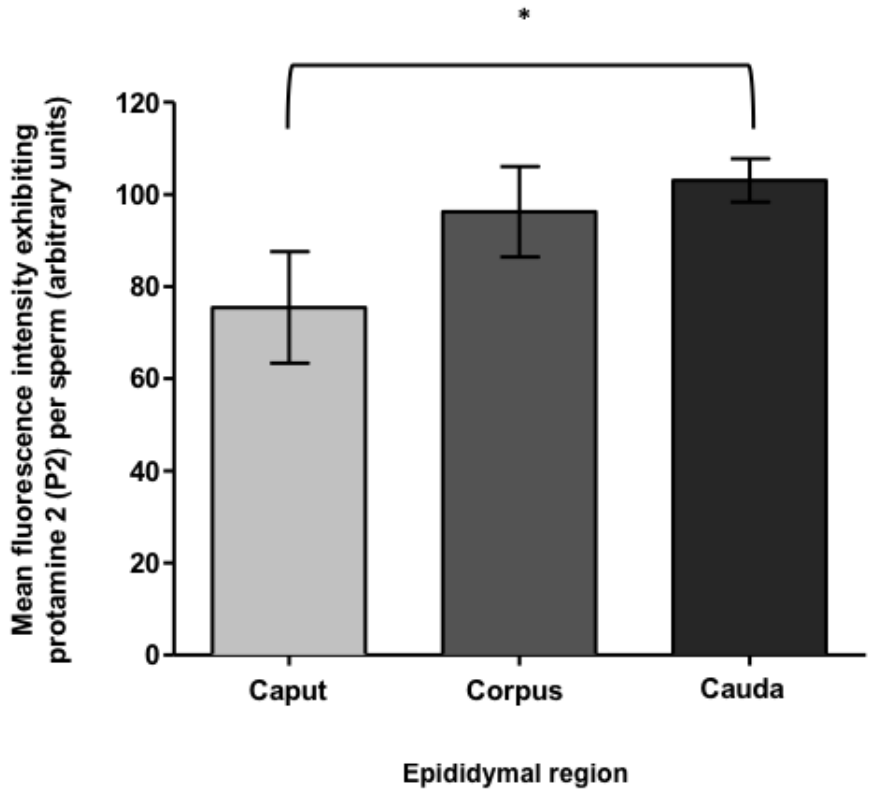


Figure 2.23: Mean relative protamine 2 (P2) fluorescence levels in sperm from caput, corpus, caudal epididymal regions of 16 week old mice (n=3). Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisks (*) denote statistical significant differences ($P \leq 0.05$) in the total levels of P2 between caput and cauda epididymal sperm. Data are shown as mean ± SEM.

The presence and localisation of P2 expressed across the sperm head are shown in **Figure 2.22**. Total levels of P2 were evaluated in a total of 300 sperm from each epididymal region. As in the case of P1, the total levels of P2 showed a gradual increase in sperm throughout the epididymal region with mean fluorescence levels of P2 of 75.5 ± 12.1AU, 96.3 ± 9.8AU and 103.0 ± 4.7AU in the caput, corpus and cauda respectively (**Figure 2.23**). The total levels of P2 in cauda sperm were significantly higher than in the caput ($P \leq 0.05$; as indicated by One-Way ANOVA).

In addition, the P1:P2 ratios in sperm from each epididymal region were evaluated by dividing the total levels of P1 by the total levels of P2 (P1/P2). The ratio obtained for caput, corpus and cauda were 0.8, 0.8, and 0.9, respectively. The P1:P2 ratio levels did not show any statistical significant differences ($P \geq 0.05$; as indicated by One-Way ANOVA) between the three epididymal regions.

2.3.4 Comparative analysis of key sperm parameters between non-cryopreserved and cryopreserved epididymal mouse sperm

The effect of cryopreservation upon sperm DNA integrity was assessed using the SCD technique in sperm from one 16 week old mouse (n=1). Since the SCD method to evaluate DNA fragmentation level was not consistent in this pilot experiment, no further mice were recruited. However, data are presented below and the results explained in terms of the differential trend observed between non-cryopreserved and cryopreserved sperm. Prior to DNA fragmentation analysis, non-cryopreserved and cryopreserved epididymal sperm from the caput, corpus and cauda regions of a 16 week old mouse (n=1) were assessed by computer assisted sperm analysis (CASA) to determine basic sperm parameters. Sperm concentration (**Figure 2.24**), motility (**Figure 2.25**), and progressive motility (**Figure 2.26**) were evaluated and compared between non-cryopreserved and cryopreserved epididymal

mouse sperm. Total motility, progressive motility and sperm concentration prior to cryopreservation increased gradually as the sperm matured through the epididymis. In contrast, cryopreserved sperm exhibited dramatically reduced levels of total motility, progressive motility and sperm concentration as compared to non-cryopreserved sperm as sperm progressed through the epididymis.

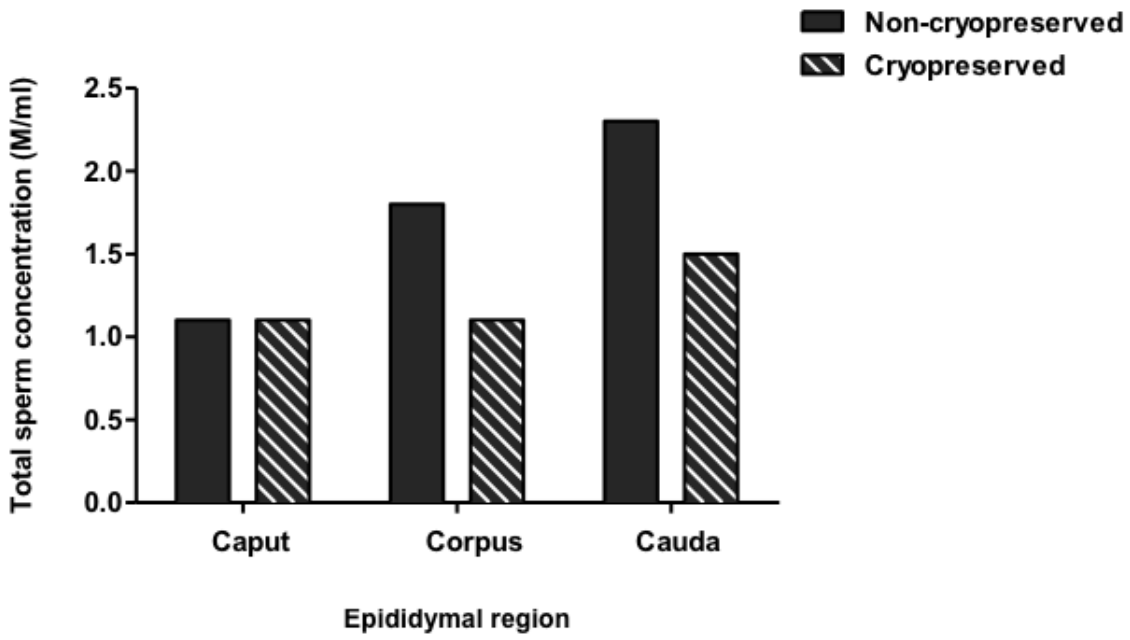


Figure 2.24: Total sperm concentration obtained from non-cryopreserved and cryopreserved epididymal sperm from a 16 week old mouse using Computer Assisted Sperm Analysis (CASA). Total sperm concentration of sperm was expressed as millions of sperm per millilitre (M/ml). No statistical analysis was possible due to n=1.

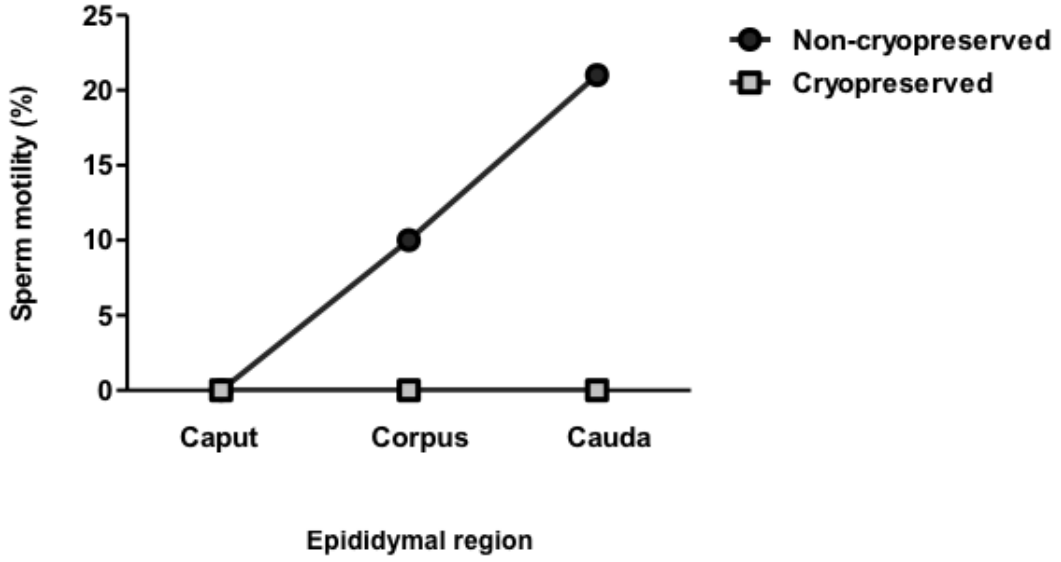


Figure 2.25: Sperm motility obtained from non-cryopreserved and cryopreserved epididymal sperm from a 16 week old mouse using Computer Assisted Sperm Analysis (CASA). Total sperm motility was expressed as a percentage. No statistical analysis was possible due to n=1.

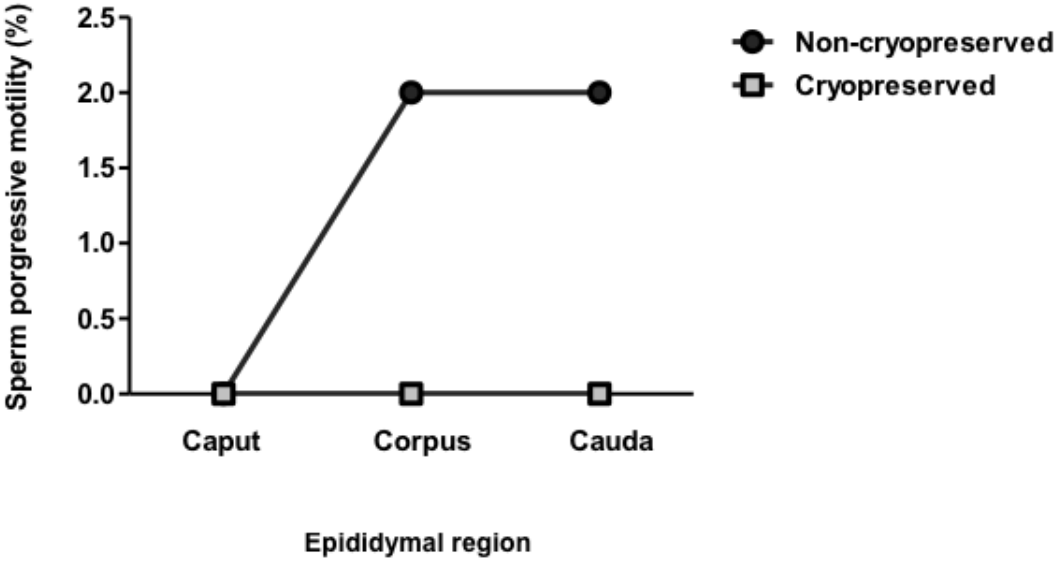


Figure 2.26: Sperm progressive motility obtained from non-cryopreserved and cryopreserved epididymal sperm from a 16 week old mouse using Computer Assisted Sperm Analysis (CASA). Sperm progressive motility was expressed as a percentage. No statistical analysis was possible due to n=1.

2.3.5 Comparative DNA fragmentation analysis between non-cryopreserved and cryopreserved epididymal mouse sperm

DNA fragmentation levels of cryopreserved sperm within the epididymis were analysed using the SCD technique. DNA fragmentation in cryopreserved sperm in comparison to non-cryopreserved sperm was measured based upon sperm head and DNA halo size which were measured using ImageJ software. Prior to measurement, the number of sperm from the caput, corpus and cauda epididymis exhibiting DNA halos following cryopreservation in comparison to non-cryopreserved sperm was evaluated. The mean proportion of sperm with fragmented DNA in cryopreserved sperm in comparison to non-cryopreserved sperm is shown in **Figure 2.27**. Prior to cryopreservation, the number of sperm exhibiting DNA fragmentation gradually decreased along the epididymis (caput, corpus and cauda) ranging from 40% - 0%. However, following cryopreservation, the proportion of sperm with fragmented DNA increased with all (100%) of the sperm analysed from the caput, corpus and cauda showing DNA fragmentation. Sperm head sizes from each epididymal region were evaluated and measured. Head size was significantly increased ($P \leq 0.05$; as indicated by Two-Way ANOVA) by 26%, 20% and 48% in caput, corpus and caudal sperm respectively following cryopreservation (**Figure 2.28**) as compared to non-cryopreserved sperm form the same locations.

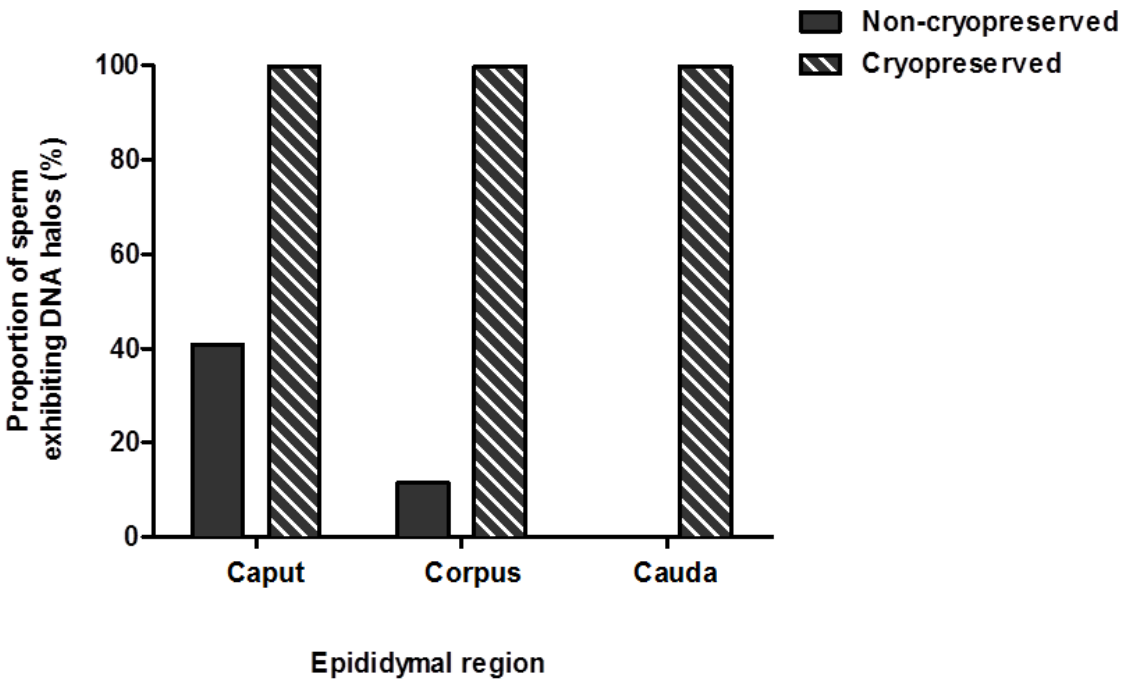


Figure 2.27: Proportion of epididymal sperm from a 16 week old mouse (n=1) exhibiting DNA halos prior to, and following cryopreservation. The number of sperm with a DNA halo was evaluated with the use of ImageJ software. Data are shown as percentages.

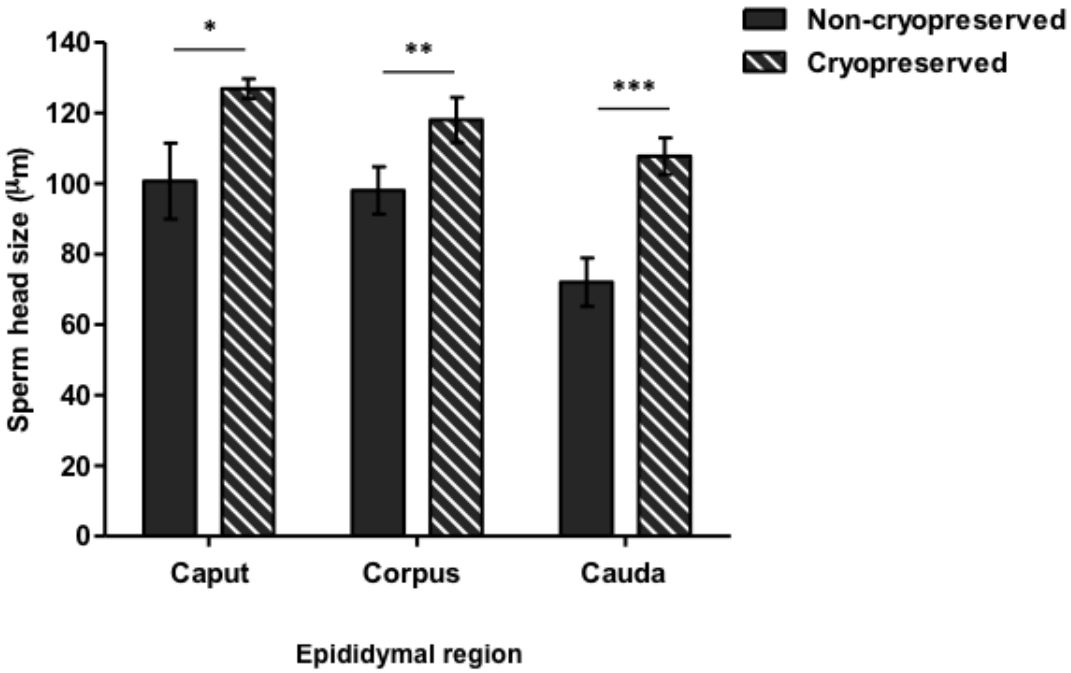


Figure 2.28: Mean sperm head size of epididymal sperm from a 16 week old mouse (n=1) prior to, and following cryopreservation. Sperm head size was measured and quantified in micrometres (µm) with the use of ImageJ software. Asterisks (*, **, ***) denote statistically significant differences ($P \leq 0.05$) between non-cryopreserved and cryopreserved sperm from caput, corpus and cauda epididymal regions respectively. Data are shown as mean ± SEM.

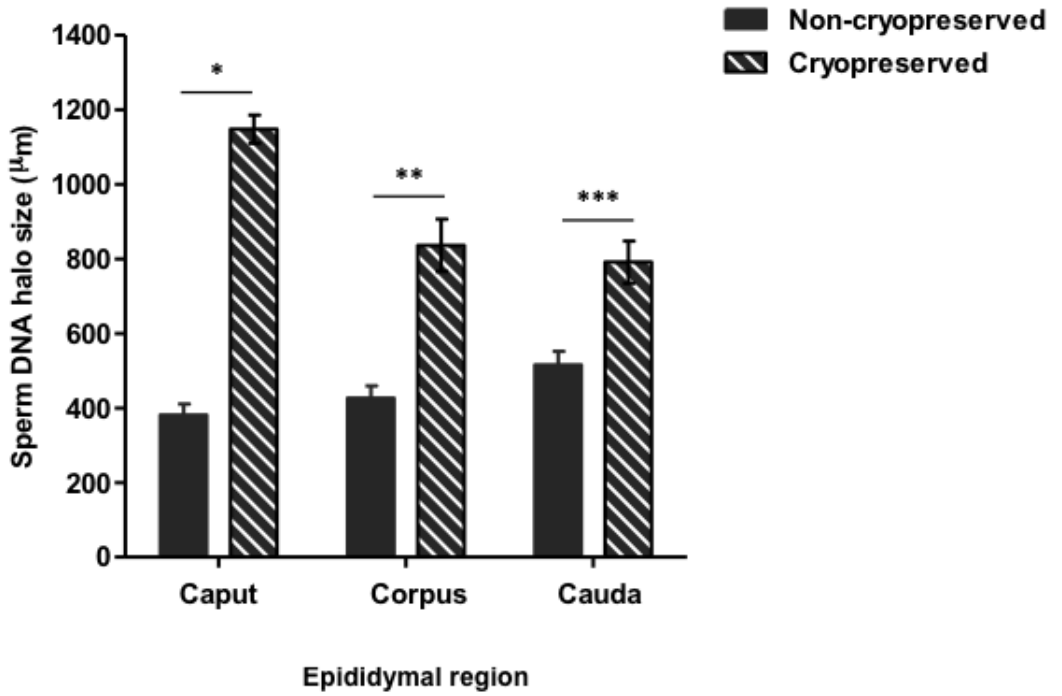


Figure 2.29: Mean DNA halo size of epididymal sperm from a 16 week old mouse (n=1) prior to, and following cryopreservation. DNA halo size was measured and quantified in micrometers (µm) with the use of ImageJ software. Asterisks (*, **, ***) denote statistically significant differences ($P \leq 0.05$) between non-cryopreserved and cryopreserved sperm from caput, corpus and cauda epididymal regions respectively. Data are shown as mean ± SEM.

Subsequently, the size of DNA halos in sperm from caput, corpus and cauda epididymal regions were evaluated and compared between cryopreserved and non-cryopreserved sperm (**Figure 2.29**). Size of DNA halo increased significantly ($P \leq 0.05$; as indicated by Two-Way ANOVA) by 200%, 96% and 53% in caput, corpus and caudal sperm respectively following cryopreservation.

2.3.6 Total levels of P1 and P2 in non-cryopreserved and cryopreserved caudal mouse sperm

Mouse caudal sperm retrieved from 16 week old mice (n=5) were assessed using CASA to determine basic sperm parameters and compared between non-cryopreserved and cryopreserved sperm. Mean concentrations of sperm were significantly higher in non-cryopreserved sperm compared to cryopreserved sperm ($P \leq 0.05$; as indicated by the Student's *t*-test) (**Figure 2.30**). Mean total motility and progressive motility of caudal sperm were significantly reduced by 60% and 20% respectively following cryopreservation ($P \leq 0.05$; as indicated by the Student's *t*-test) (**Figure 2.31**).

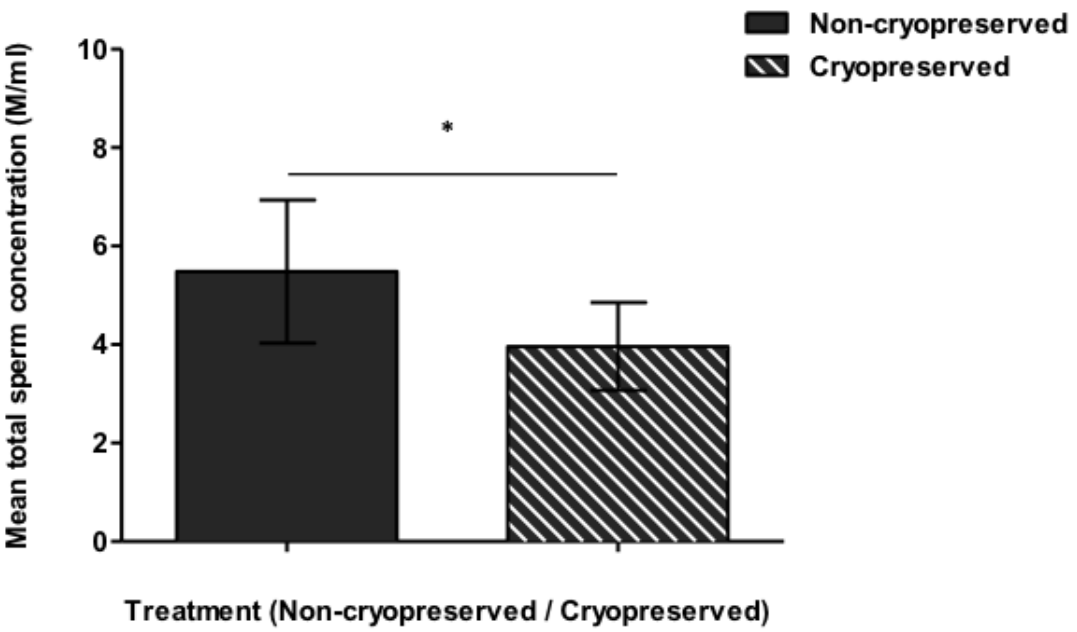


Figure 2.30: Mean total sperm concentration obtained from non-cryopreserved and cryopreserved caudal sperm of 16 week old mice (n=5) using Computer Assisted Sperm Analysis (CASA). Total sperm concentration was expressed as millions of sperm per millilitre (M/ml). Asterisk (*) denotes a statistically significant difference ($P \leq 0.05$) in the total sperm concentration between non-cryopreserved and cryopreserved caudal sperm. Data are show as mean ± SEM.

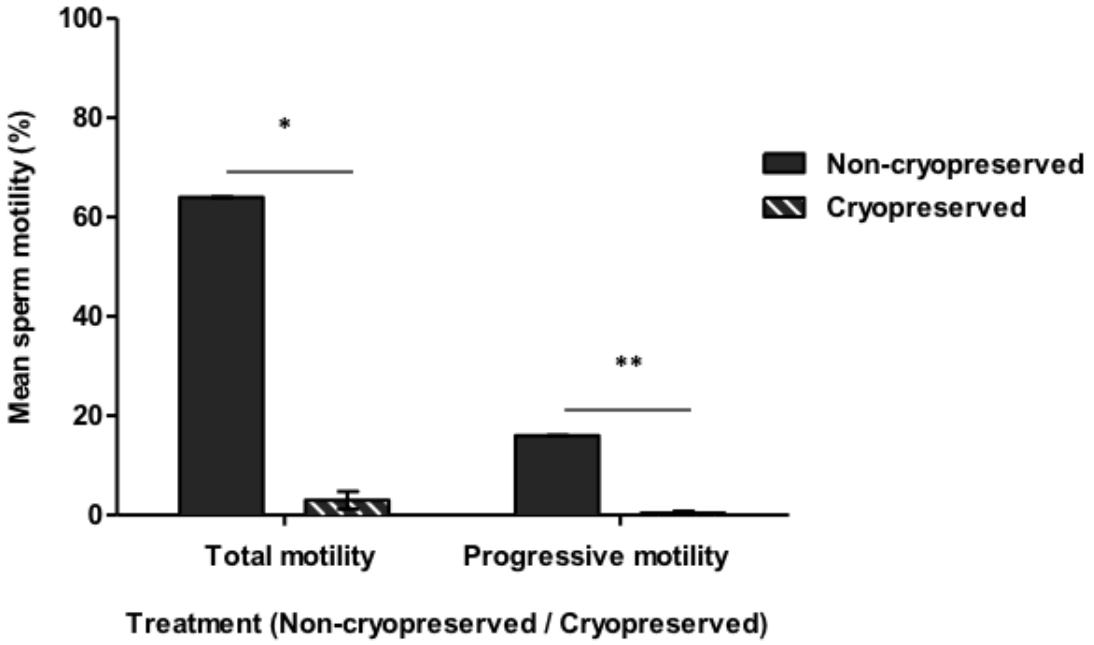


Figure 2.31: Mean sperm motility obtained from non-cryopreserved and cryopreserved caudal sperm of 16 week old mice (n=5) using Computer Assisted Sperm Analysis (CASA). Total sperm motility and sperm progressive motility was expressed as percentages. Asterisks (***, ****) denote statistically significant differences ($P \leq 0.05$) between non-cryopreserved and cryopreseved caudal sperm. Data are show as mean ± SEM.

The localisation of P1 (**Figure 2.32A**) and P2 (**Figure 2.33A**) in both non-cryopreserved and cryopreserved mouse caudal sperm are presented in representative confocal microscopic images. The reduced fluorescence intensity levels and punctuated form of P1 (**Figure 2.32B**) and P2 (**Figure 2.33B**) were clearly demonstrated in cryopreserved sperm as compared to non-cryopreserved sperm. Following immunofluorescence quantification, the total levels of P1 and P2 in cryopreserved sperm were significantly reduced as compared to non-cryopreserved sperm. The total number of sperm analyzed to evaluate the total levels of P1 and P2 were 450 and 442 for non-cryopreserved sperm, and 351 and 340 for cryopreserved sperm respectively.

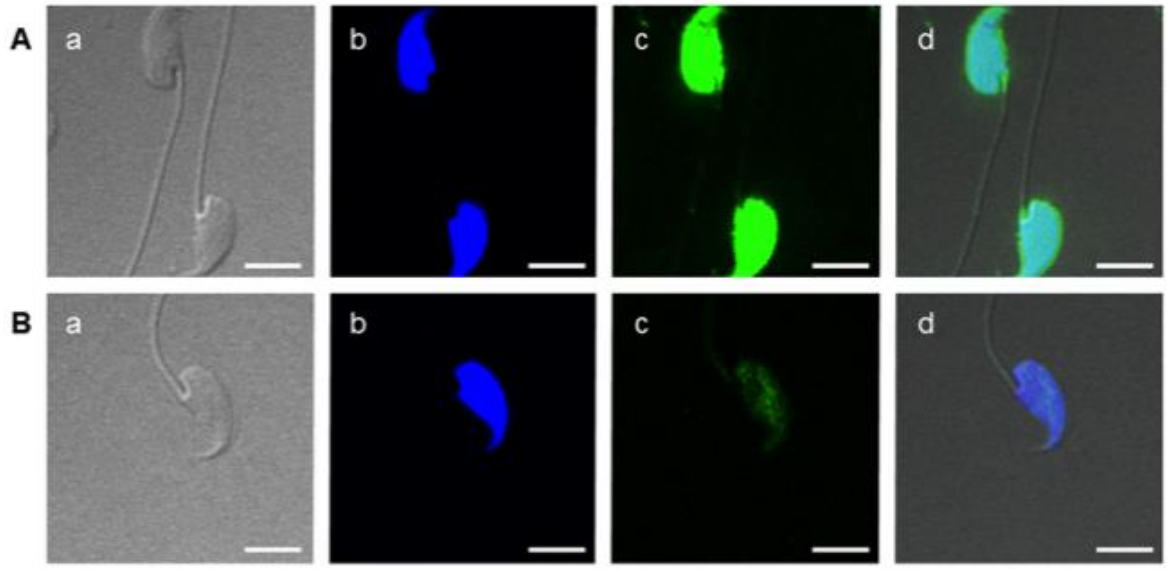


Figure 2.32: Confocal images of (a) bright-field (b) nuclei, (c) protamine 1 (P1), and (d) overlay of mouse caudal sperm. Figure shows the presence of P1 (**A**) prior to cryopreservation and (**B**) much reduced levels of P1 following cryopreservation. Image captured at x100 magnification. White scale bar indicates 3.5µm.

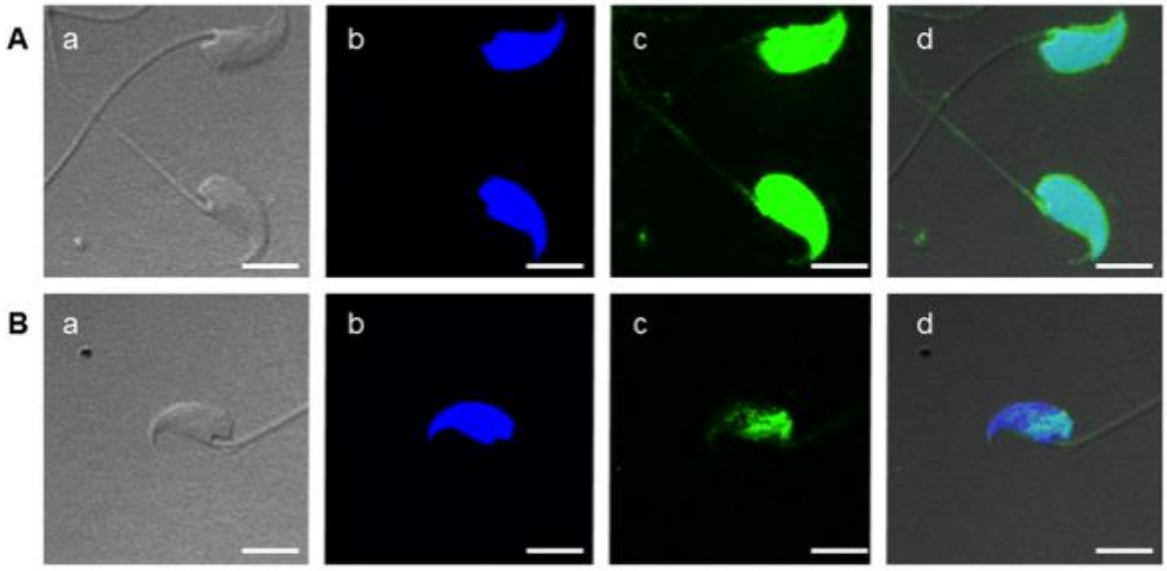


Figure 2.33: Confocal images of (a) bright-field (b) nuclei, (c) protamine 2 (P2), and (d) overlay of mouse caudal sperm. Figure shows the presence of P2 (**A**) prior to cryopreservation and (**B**) much reduced levels of P2 following cryopreservation. Image captured at x100 magnification. White scale bar indicates 3.5µm.

The total levels of P1 in cryopreserved sperm (52.2 ± 9.9AU) were significantly reduced ($P \leq 0.05$; as indicated by the *t*-test) by 45% as compared to non-cryopreserved sperm (94.5 ± 9.5AU) (**Figure 2.34**). Similarly, the total levels of P2 in sperm were significantly lower by 52% following cryopreservation (46.1 ± 8.9AU) as compared to non-cryopreserved sperm (95.4 ± 10.4AU) (**Figure 2.34**). Furthermore, protamine ratios were determined by dividing the total levels of P1 by the total levels of P2, (P1/P2). The resulting P1:P2 ratios obtained were 1.0 and 1.1 for non-cryopreserved and cryopreserved sperm respectively. The data thus obtained a slight increase of P1:P2 ratio in cryopreserved sperm as compared to non-cryopreserved sperm.

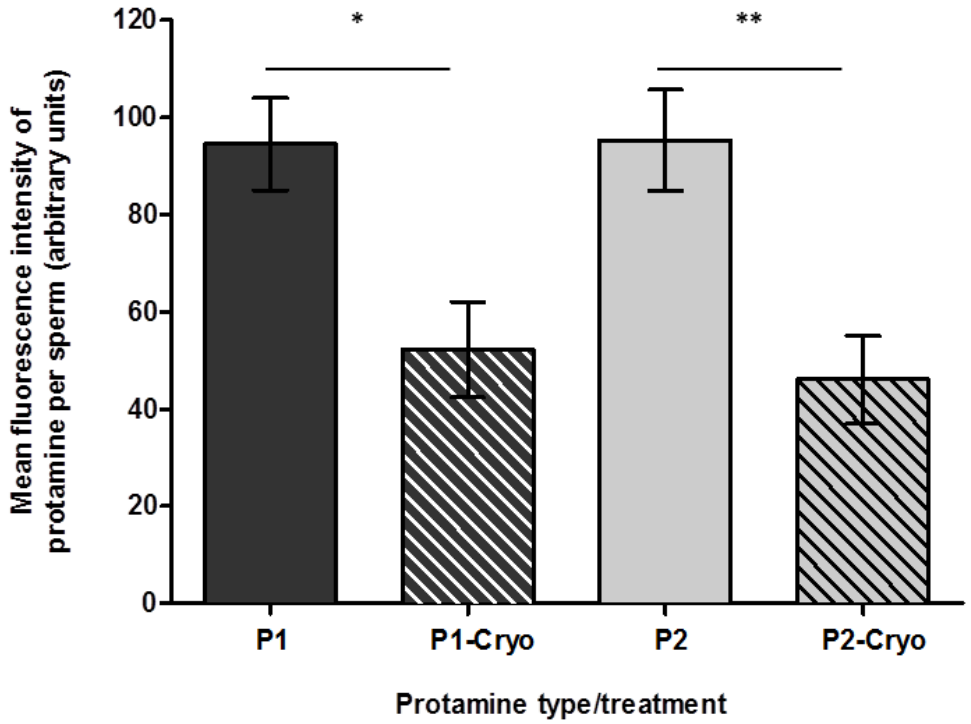


Figure 2.34. Mean relative protamine 1 (P1) and protamine 2 (P2) fluorescence levels in sperm from the caudal region of 16 week old mice (n=5), prior to, and following cryopreservation. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisks (***,****) denote statistically significant differences ($P \leq 0.05$) in the total levels of protamine between non-cryopreserved and cryopreserved sperm. Data are shown as mean ± SEM.

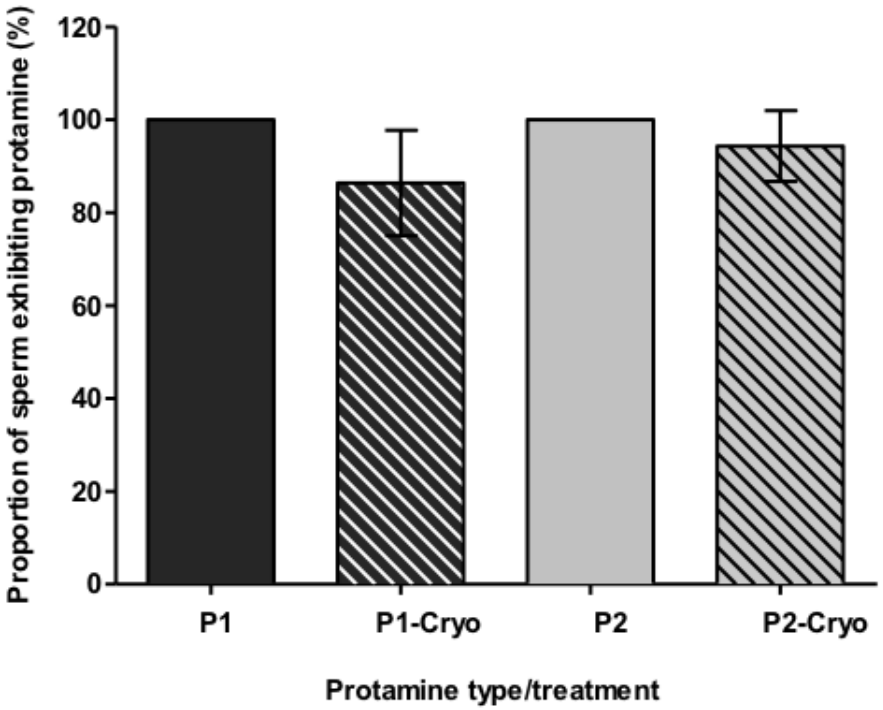


Figure 2.35: Mean proportion of caudal sperm from 16 week old mice (n=5) containing protamine 1 (P1) and protamine 2 (P2) prior to, and following cryopreservation. Number of sperm with protamine was determined with the use of ImageJ software. There was no statistically significant differences in the number of sperm containing P1 or P2 between non-cryopreserved and cryopreserved sperm. Data are shown as percentages.

Proportional analysis performed to evaluate the number of cryopreserved mouse sperm containing P1 and P2 in comparison to non-cryopreserved sperm showed a non-significant reduction ($P \geq 0.05$; as indicated by the Student's *t*-test). For example, the total number of non-cryopreserved sperm analyzed for P1 (n= 450) and P2 (n=442) showed the presence of P1 and P2 in all sperm (100%) (**Figure 2.35**). In the cryopreserved sperm, 7% and 6.5% appeared to be completely devoid of P1 and P2 respectively. Although, the number of sperm that were devoid of P1 and P2 were low, most cryopreserved sperm showed a punctuated localisation pattern of protamine (**Figure 2.32B**) and (**Figure 2.33B**) with much lower fluorescent intensity (**Figure 2.34**).

2.4 Summary of results

1. Specific primary antibodies for P1 and P2 were selected and optimised for concentration. Secondary concentration was also optimised and the P1 and P2 antibodies were tested for specificity.
2. A novel quantitative immunofluorescent assay was successfully developed for P1 and P2 in mouse sperm, which provided information on expression level and spatial localisation.
3. Sperm concentration, total motility and progressive motility increased gradually and significantly as sperm matured along the mouse epididymal tract.
4. Total levels of P1 and P2 increased significantly as sperm matured within the mouse epididymis, and were localized across the entire sperm head.
5. P1:P2 ratio did not differ when compared between the caput, corpus and cauda epididymis.
6. Sperm concentration, total motility and progressive motility were significantly reduced following the cryopreservation of mouse sperm.
7. DNA fragmentation levels (based upon the expansion of sperm head size and DNA halo size) was significantly higher in caput sperm compared to corpus and caudal sperm following cryopreservation suggesting that the least mature sperm were most vulnerable to cryo-injury.
8. Total levels of P1 and P2 were significantly reduced in sperm that had been cryopreserved/thawed sperm compared to non-cryopreserved sperm. Localisation of P1 and P2 in cryopreserved sperm appeared to be punctuated.
9. P1:P2 ratios were comparable between non-cryopreserved and cryopreserved sperm and were not statistically different.

2.5 Discussion

In the current chapter, I investigated whether routine clinical procedures such as cryopreservation could detrimentally affect the expression and localisation of the key-sperm nuclear protein, protamine. Previously, Kashir *et al*. (2011a) reported that the total levels of PLCζ, a sperm protein vital for oocyte activation were significantly reduced in cryopreserved sperm from fertile control men. This finding led us to expand our investigations to investigate the potential effect of cryopreservation upon protamine, a nuclear protein which plays a critical role in establishing, maintaining and protecting paternal genomic DNA upon fertilisation. There have been no other published reports assessing the potential adverse effects of cryopreservation upon the total levels of protamine. Moreover, protamine deficiency in human sperm has been strongly linked with male infertility via poor sperm parameters, reduced sperm penetration ability, increased sperm DNA fragmentation and embryonic death leading to poor IVF or ICSI outcome, including total fertilisation failure.

The specific mechanism involved in aberrant protamine content and ratio in relation to infertility remains unknown, however, abnormalities during spermatogenesis, alterations in gene expression, mutations and SNPs in the protamine gene can all be attributed to alterations in protamine ratio (Francis *et al.*, 2014). Moreover, DNA fragmentation mechanisms, oxidative stress attributed to increased levels of ROS during spermatogenesis, and post-testicular transport may also influence abnormalities in protamine content and ratio (Sakkas & Alvarez, 2010).

Thus far, very little is known about whether external or clinical factors could potentially affect protamine levels in sperm. Therefore, in this chapter, I evaluated the levels of protamine in epididymal mouse sperm prior to and following cryopreservation. It is well known that cryopreservation could detrimentally affect sperm DNA integrity, therefore DNA

fragmentation analysis in epididymal mouse sperm was carried out prior to and following cryopreservation as a preliminary experiment. This experiment was essential because there is clear association between DNA fragmentation and protamine deficiency in human sperm. Thus, results obtained from this experiment provided a link to the next aim to investigate the effect of cryopreservation upon the total levels of protamine in mouse sperm. This investigation was carried out in mouse sperm to test the hypothesis that cryopreservation may cause detrimental effects upon total levels of protamine. To our knowledge, this is the first study to utilise immunofluorescent techniques to perform such an investigation. In future, such studies should be extrapolated to the human scenario. I also aimed to determine whether immunofluorescent methods could be used as a potential clinical tool with which to evaluate protamine levels in human sperm to select the best sperm for ART. Prior to performing the protamine immunofluorescent investigations, I first validated and evaluated the specificity of the monoclonal protamine antibodies in mouse and boar sperm. Boar sperm was specifically used for specificity testing as it contains only P1 and not P2. Therefore, P2 antibody should not show any binding in boar sperm. The immunoreactivity and specificity of the two protamine antibodies were successful proven in boar sperm and mouse sperm. Sperm parameters were also evaluated using CASA and compared between non-cryopreserved and cryopreserved mouse sperm.

2.5.1 Total levels of P1 and P2 in epididymal mouse sperm

Protamine plays a vital role during epididymal maturation, which includes cross-linking between protamine molecules and sperm nuclear chromatin to form disulfide bridges (Oliva, 2006). Moreover, the phosphorylation of protamine, which further contributes to the compaction of sperm nuclear DNA, is also known to occur during this stage (Oliva, 2006; Balhorn, 2007; Francis *et al.*, 2014).

This part of the study aimed to investigate basic sperm parameters in sperm retrieved from different parts of the epididymis. Sperm progressively matures with increasing membrane integrity and development as they move through the epididymis (Cooper, 2007). In support to these mechanisms, CASA data obtained in the present study showed increasing concentration, motility and progressive motility from the caput to the cauda.

Next, I investigated whether levels of P1 and P2 differ in mouse sperm during the epididymal maturation process. It has been shown that during post-testicular transport, sperm can be exposed to ROS or oxidative damage within the epididymis leading to DNA fragmentation and abnormal protamination (Cocuzza *et al.*, 2007; Tremellen, 2008; El-Taieb *et al.*, 2009; Sakkas & Alvarez, 2010). Therefore, it is important to identify if such effects may lead to protamine deficiency. Following immunofluorescence analysis, total levels of P1 and P2 increased steadily and significantly as sperm progressed through the epididymis. Formation of disulfide bonds and cross-linking during protamine packaging do not only protect sperm from DNA damage, or the effects of endonucleases and ROS, but also prevent the disassociation of protamine from DNA. Increasing levels of protamine in sperm progressing through the epididymis may confirm the importance of protamine disulfide cross-linking during epididymal maturation. Moreover, P1:P2 ratios in maturing sperm were comparable between each epididymal region indicating that there were no detrimental factors affecting total levels of P1 and P2 during the post-testicular transport of mouse sperm.

Collectively, data showed a clear association between increasing sperm motility and increasing protamine levels during epididymal maturation indicating the fundamental role of protamine for sperm to establish optimal motility and fertilisation ability. These findings are limited only to mouse sperm and it is difficult to extrapolate such findings to human sperm, especially since human sperm would be acquired by ejaculation and not swim-out. It will also be interesting to evaluate protamine levels and localisation in human sperm retrieved from

testicular biopsies using the immunofluorescent method developed herein and assess whether this technique may be a potential novel diagnostic tool for clinical application.

2.5.2 Comparative analysis of key sperm parameters between non-cryopreserved and cryopreserved mouse sperm

Sperm cryopreservation is a well-established technique often used to preserve the sperm of men who are subjected to certain medical treatments such as chemotherapy/radiotherapy or surgery (Di Santo *et al.*, 2012). Cryopreservation is also used to preserve sperm retrieved from surgical sperm retrieval (SSR) cases or testicular biopsies in order to prevent repetitive biopsies (Gangrade, 2013). Moreover, sperm banking and sperm donor programs rely heavily upon cryopreservation (Di Santo *et al.*, 2012). While the benefit of sperm cryopreservation is clear, the detrimental effect of this procedure upon sperm parameters, structure, function, DNA integrity, and key sperm proteins is undeniable, and requires significant investigation in future.

CASA consistently showed lower sperm concentration, motility, and progressive motility in cryopreserved compared to non-cryopreserved mouse sperm. Cryopreservation is known to cause adverse effects upon sperm via osmotic shock and cell shrinkage (Isachenko *et al.*, 2004a), thus compromising sperm motility and viability (Donnelly *et al.*, 2001b; Gadea *et al.*, 2011). It is most likely that the present data showing a clear detrimental effect of cryopreservation upon the concentration and motility of progressive sperm were caused by such mechanisms. Previous work has shown that cryopreservation can cause detrimental effects upon several sperm parameters such as motility, concentration, viability and morphology (Vutyavanich *et al.*, 2010; Di Santo *et al.*, 2012) and can cause deleterious effects upon sperm nuclear chromatin, which may subsequently promote DNA fragmentation

(Zribi *et al.*, 2010). The effect of cryopreservation upon DNA integrity of sperm is discussed further in **Section 2.5.3**.

The current findings are generally similar to those previously reported (Zribi *et al.*, 2010; Gadea *et al.*, 2011; Boitrelle *et al.*, 2012) but extend such studies by providing novel information relating to progressive sperm – considered to be the 'fittest' sperm that are most likely to fertilise. The adverse effects of cryopreservation upon sperm quality are clearly evident in the present results. Since this laboratory technique is widely use in ART, it follows that modifications to existing protocols, or the development of new techniques, may help to improve the quality of sperm prior to ART. Given the clear effects upon the concentration and motility of progressive sperm, it seems highly plausible that other parameters and mechanisms such as sperm viability and key sperm proteins, are also susceptible to freeze-thaw damage and that future research should be directed to a better understanding of such effects. The detrimental effect of cryopreservation upon sperm DNA integrity and protamine contents are described in **Sections 2.5.3** and **2.5.4** respectively.

2.5.3 Comparative DNA fragmentation analysis between non-cryopreserved and cryopreserved epididymal mouse sperm

Mounting evidence supports the adverse effect of cryopreservation upon DNA integrity in sperm. Indeed, several studies have reported increased DNA fragmentation levels in cryopreserved compared to non-cryopreserved sperm (Donnelly *et al.*, 2001a; Gandini *et al.*, 2006; Yildiz *et al.*, 2007; Ahmad *et al.*, 2010). In line with these studies, the current data demonstrated an increased proportion of sperm exhibiting DNA fragmentation following cryopreservation, based upon measurement of the sperm head and DNA halos. Moreover, DNA fragmentation analysis based upon DNA halo measurement within epididymal sperm

indicated vulnerability of the least mature sperm (caput sperm) to cryo-injury compared to the most matured sperm (cauda).

DNA fragmentation in epididymal mouse sperm prior to and following cryopreservation was assessed using the SCD method. Firstly, DNA fragmentation levels were analysed in sperm retrieved from the three epididymal regions prior to cryopreservation in order to determine the susceptibility of such sperm to DNA damage. Expansion of the sperm head and DNA halo following cryopreservation is a known indicator of DNA fragmentation level. As demonstrated previously by Perez-Cerezales *et al*. (2012) and in the present study, the number of sperm exhibiting DNA halos was higher in caput sperm compared to corpus and caudal sperm, thus indicating that the least mature sperm are more vulnerable to DNA damage (Perez-Cerezales *et al.*, 2012). DNA fragmentation levels (as determined by the size of sperm DNA halos) decreased along the epididymis indicating that the more mature the sperm become (eg. in the cauda), the more resistant they were to cryo-injury.

During post-testicular transport, the sperm undergoes a maturational process, which mainly involves the development of sperm membrane integrity within the epididymis prior to ejaculation (Perez-Cerezales *et al.*, 2012). Protamine plays a vital role during epididymal maturation, which includes cross-linking between protamine molecules and sperm nuclear chromatin to form disulfide bridges (Oliva, 2006). It is known that sperm are vulnerable to DNA damage and abnormal protamination due to exposure to ROS and oxidative stress in the epididymus (Sakkas & Alvarez, 2010). Since sperm mature as they progress from the caput, corpus to cauda epididymides (Cooper, 2007), the least mature sperm are expected to be more susceptible to DNA damage as compared to corpus or caudal sperm. Collectively, these mechanisms are likely to have contributed to the increased number of sperm exhibiting DNA halos observed in caput sperm compared to those from the corpus or cauda.

Secondly, DNA fragmentation levels were compared between non-cryopreserved and cryopreserved epididymal mouse sperm. Proportional analysis indicated that the numbers of epididymal sperm exhibiting DNA halos following cryopreservation were higher compared to non-cryopreserved sperm. Moreover, DNA fragmentation levels (based upon sperm DNA halo size) were higher in caput sperm in comparison to other epididymal regions following cryopreservation. These findings also suggest that the least mature sperm exhibiting poor membrane integrity are likely to be more vulnerable to cryo-damage.

Collectively, data from this experiment clearly indicated that cryopreservation could influence sperm DNA integrity, as demonstrated previously (Donnelly *et al.*, 2001b; Boitrelle *et al.*, 2012; Di Santo *et al.*, 2012; Valcarce *et al.*, 2013). The low number of samples used in the present study may of course limit the strength of the results obtained and it would be useful to extrapolate these studies to human sperm with a larger number of samples. It is difficult to extrapolate the present findings to epididymal human sperm. However, the higher proportions of DNA fragmented sperm seen in the least mature sperm may also indicate that sperm from human testicular biopsies are also vulnerable to DNA fragmentation. Moreover, such effects may be even greater in testicular biopsy patients who choose to cryopreserve their sperm to avoid repetitive biopsies. It is crucial for future studies to recruit human sperm samples and surgically retrieved sperm to evaluate DNA fragmentation levels and whether cryopreservation could detrimentally affect the DNA integrity of such sperm.

2.5.4 Total levels of P1 and P2 in non-cryopreserved and cryopreserved sperm from the mouse cauda epididymus

Previous studies have demonstrated that sperm from infertile men that exhibits elevated levels of DNA fragmentation also showed abnormalities in protamine content or alterations in protamine ratio (Aoki *et al.*, 2005a; Simon *et al.*, 2011). In addition, such sperm

exhibits abnormal motility, morphology and concentration as compared to sperm from fertile men (Aoki *et al.*, 2005a; Aoki *et al.*, 2006a). The detrimental effect of cryopreservation upon DNA fragmentation has been demonstrated previously by Donnelly *et al*. (2001a) and Yildiz *et al*. (2007). In the present study, I investigated whether cryopreservation could also alter protamine levels and P1:P2 ratio in sperm. Although, many studies have shown increased DNA fragmentation levels in cryopreserved sperm (Gandini *et al.*, 2006; Ahmad *et al.*, 2010), nothing is known of how cryopreservation may influence total levels of protamine. A recent study compared DNA fragmentation in cryopreserved sperm from eleven mammals and indicated that the specific structural presentation of P1 and P2 correlated with chromatin stability and created resistance to cryo-injury induced DNA damage (Gosalvez *et al.*, 2011). However, this earlier study did not evaluate protamine levels or protamine ratio. Several other immunofluorescent and Western blot studies have demonstrated the detrimental effect of freeze/thaw upon nucleoprotein structure, disulfide bridges, and interaction between protamine 1 and DNA in boar sperm (Flores *et al.*, 2008; Flores *et al.*, 2011). Thus far, such studies have been limited to boar sperm only and to our knowledge, there has been no such investigations carried out on protamine levels in mouse or human sperm via electrophoresis or immunocytochemistry following cryopreservation.

In the current chapter, a comparative study between non-cryopreserved and cryopreserved mouse sperm was carried out using immunofluorescent cytochemistry staining to directly assess the total levels of protamine and P1:P2 ratio. Findings from these analyses clearly demonstrated significantly reduced levels of P1 and P2 in cryopreserved sperm compared to non-cryopreserved sperm. Interestingly, P1:P2 ratio was higher in cryopreserved sperm than non-cryopreserved sperm. Alteration in P1:P2 ratio, and insufficiency in protamine content of sperm, has been associated with male infertility, poor fertilisation rate and embryonic death (Cho *et al.*, 2003; Depa-Martynow *et al.*, 2012). Consequently, the

current data do not only indicate the detrimental effect of cryopreservation upon the total levels and ratio of protamine, but also highlight the possibility that cryopreserved sperm from either fertile or infertile men may exhibit dysfunctional ability for fertilisation and normal embryonic development. P1 and P2 in sperm nuclei are organised in a species-specific manner due to evolutionary variation (Corzett *et al.*, 2002; Balhorn, 2007; Gosalvez *et al.*, 2011). This has also led to variations of P1:P2 ratio in sperm across various species. For example, P1:P2 ratio in mouse sperm is 1:2 or 0.5 according to acid-urea gel electrophoresis and high performance liquid chromatography (HPLC) techniques respectively (Corzett *et al.*, 2002). In mouse sperm, P1 is made up of 50 amino acids while P2 is made up of 106. However, towards the final stage of sperm maturation, P2 undergoes proteolysis and 40% of the amino acids are removed resulting in a 63 amino acid P2 protein in the mature sperm head (Balhorn, 2007). Given that the current findings demonstrate a P1:P2 ratio ranging between 1.0 to 1.1, such findings may be related to an approximate equal proportion for P1 and P2 in mature mouse sperm. This finding differs slightly from a previous report stating that P1:P2 ratio in mouse sperm is 1:2 (Corzett *et al.*, 2002). However, in the current study, P1 and P2 were evaluated separately and in individual mouse sperm by immunofluorescent methodology, rather than the electrophoresis and HPLC method used by Corzett *et al*. (2002). The analysis of both P1 and P2 simultaneously in each individual sperm may allow better P1 and P2 levels and thus provide a more accurate ratio. Moreover, protamine ratio has been shown to exhibit large variation, particularly when evaluated by the acid-urea gel electrophoresis technique (Nanassy *et al.*, 2011). Since, the protamine ratio observed in the present study did not concur with the expected ratio given by earlier reports, a second study was carried out using acid-urea gel electrophoresis to assess whether methodology influenced data outcome (described in Chapter 3).

Previous reports have demonstrated that sperm exhibiting an abnormal P1:P2 ratio have poor penetration ability into the oocyte, thus explaining low fertilisation rates of such sperm following IVF but not ICSI (Aoki & Carrell, 2003; Aoki *et al.*, 2005b). However, concern remains with regards to the long-term effect upon offspring, such as genetic abnormalities due to the use of sperm with abnormal protamination/fragmented DNA sperm for ICSI (Fernandez-Gonzalez *et al.*, 2008; Francis *et al.*, 2014). In addition to the current findings, which indicated the detrimental effect of cryopreservation upon total levels of P1 and P2 in sperm, it is prudent to consider whether utilisation of the novel immunofluorescence method described herein to evaluate total levels of protamine in individual sperm may represent a potential biological tool with which to evaluate sperm quality and DNA integrity and thus to predict the functional ability of a sperm. While basic sperm parameter and DNA fragmentation testing are already clinically available, the evaluation of protamine ratio may also serve as beneficial additional clinical tool for male infertility, especially given published evidence suggesting that sperm penetration ability is dependent upon protamine content or ratio (Aoki & Carrell, 2003; Aoki *et al.*, 2005b). Previous studies have indicated a large variation in P1:P2 ratio among fertile men. Collective analysis of P1:P2 ratio from all previous studies led to the development of a new P1:P2 ratio of 0.54-1.43 in fertile men for clinical consideration (Nanassy *et al.*, 2011). The use of protamine mRNA ratio has also been suggested for clinical utilisation (Rogenhofer *et al.*, 2013). The existing direct assessment method bases upon gel electrophoresis and mRNA quantification to evaluate protamine ratio may however be considered as time consuming and expensive. Therefore, the novel immunofluorescent staining technique developed herein for the direct evaluation of protamine levels or ratio may represent a cost effective and efficient clinical alternative. Moreover this method may also be useful to evaluate protamine levels in SSR sperm, since gel electrophoresis requires at least 10-20M/mL of sperm for analysis.

Prior to the implementation of such a biological assay for clinical use, it would be necessary for future studies to first extrapolate such findings to human sperm and further investigate the functional ability (eg. ability to fertilise and create a good quality embryo) of cryopreserved sperm in comparison to non-cryopreserved in line with the evaluation of protamine levels and P1:P2 ratio.

2.6 Summary and future directions

Alterations in protamine content and P1:P2 ratio have been clearly associated with male infertility including abnormal sperm parameters, DNA integrity, sperm penetration ability and embryonic death. The effect of such abnormalities may also contribute to the increased incidence of genetic defects in children born following ICSI (Amor & Halliday, 2008; Hiura *et al.*, 2012). While protamine content and DNA integrity of a sperm plays a major role in successful fertilisation and embryonic development, the risk of abnormalities occurring during spermatogenesis leading to poor protamination and increased DNA fragmentation in sperm from infertile men are unavoidable. Moreover, the potential effect of iatrogenic damage such as cryopreservation upon the total levels of protamine and sperm DNA integrity in fertile or infertile men can be linked to the failure of IVF/ICSI (Yelumalai *et al.*, 2012), although sperm from infertile men may exert higher susceptibility to such damage. Collectively, the present findings suggest that the protamine evaluation method developed herein could be a useful novel biomarker to predict problems associated with sperm DNA integrity. However, the limitation of the current study is that there were no functional studies such as ICSI or IVF performed and that extrapolation of such findings to human sperm has yet to be carried out. Future studies should focus upon these shortfalls.

It is evident that sperm exhibiting protamine deficiency have poor DNA integrity. The utilisation of sperm with poor DNA integrity for ART may lead to pre-mature sperm de-

condensation following gamete fusion. However, this may not necessarily end up in fertilisation failure. ICSI is a highly efficient technique with a mean fertilisation rate of approximately 80%. ICSI can treat most types of male infertility including OAD, morphological defects, and can utilise testicular sperm or even non-viable sperm (Stecher *et al.*, 2011). This also may also inadvertently include sperm with poor DNA integrity or protamine deficiency as shown in the present study, although functional studies are still required to confirm our findings.

The consequence of ICSI in relation to embryo health and long-term genetic or epigenetics effects upon offspring health is poorly understood and remains a significant concern. For example, one particular study using a mouse model demonstrated that ICSI using DNA fragmented mouse sperm reduced the rates of embryonic development and implantation rate, and led to reduced litter size. Surviving offspring aged prematurely and exhibited a short life span. Moreover, some of the offspring developed behavioral problems and mesenchymal tumors (Fernandez-Gonzalez *et al.*, 2008). Another study showed that children born following ICSI with testicular and epididymal-retrieved human sperm developed congenital and cardiac malformations (Fedder *et al.*, 2013). A recent study by Sandin *et al*. (2013) reported an increasing number of children born following ART treatments with Autism and mental retardation (Sandin *et al.*, 2013a). Moreover, the prevalence of epigenetic abnormalities disease such as Angelman, Beckwith-Wiedemann and Silver Russell syndromes are frequently observed in children born via ART (Amor & Halliday, 2008).

Collectively, these findings suggest that ICSI may exert long-term effects upon the health of children, and this may be predominantly attributed to deficiency in the fertilising sperm. In the present study, cryopreservation was demonstrated to reduce the levels of protamine in sperm, which could lead to poor DNA integrity and the use of such sperm in

ART may be highly questionable if risk can be demonstrated to disease and abnormality in offspring. The effect of cryopreservation upon the levels of protamine in healthy mouse sperm retrieved via swim-out also suggests that such an effect may be greater in unhealthy or infertile patient sperm obtained via testicular biopsy. Immunofluorescent screening of protamine may therefore represent a reliable tool to assess the quality and integrity of sperm prior to treatment. However, extensive clinical studies based on the current findings are required prior to the clinical application of such a tool for diagnostic purposes. Development of this type of tool could also be beneficial for protamine assessment in cryopreserved donor sperm. Another, very different consideration is that research institutes forming upon mouse genetics, such as the Medical Research Council (MRC) in Harwell produce a multitude of genetically altered mouse lines and hold large banks of frozen sperm from each line as a research source. The present findings indicate that the routine cryopreservation of such sperm cause detrimental affects to the sperm DNA thus potentially compromising subsequent experiments. Furthermore, animal sperm in veterinarian institutes are usually cryopreserved for research purposes and prior to insemination to increase animal production for meat supply. It is possible therefore that the use of cryopreserved sperm may also affect the genetic health of offspring and the quality of meat produced. The consequences of such effects in food production and upon the health of consumers remain unknown.

In summary, although the present study indicated several fundamental outcomes based on sperm DNA integrity and protamine assessment in a mouse model, such findings need to be urgently extrapolated to human sperm. This would provide a stronger clinical link and may allow improvement of current ART procedures. Importantly, this study also developed novel immunofluorescent method for protamine assessment in individual sperm, which may serve as a potential diagnostic tool for clinics to predict the genetic health of a sperm, which may help to increase ART success rates.

Chapter 3

The effect of cryopreservation upon the total levels and ratio of the sperm nuclear protein, protamine (P1 & P2) in mouse sperm:

A direct protamine quantification method using acid-urea gel electrophoresis

3.1 Introduction

The fundamental role of protamine, the sperm nuclear protein and the significance of total levels of P1, P2 and P1:P2 ratios in association with various aspects of male factor infertility were described extensively in Chapter 2. Moreover, the detrimental effects of cryopreservation upon the total levels of P1 and P2 in mouse sperm were clearly demonstrated via an immunofluorescence technique, and the consequences of such effects were discussed.

Thus far, protamine levels and P1:P2 ratios in sperm from infertile men in comparison to fertile men have been routinely quantified using a direct assay known as acid-urea gel electrophoresis (Oliva, 2006). The first study to demonstrate abnormal protamine content using this method was reported by Silvestroni *et al*. (1976) who described the retention of histones in sperm from a large group of infertile men with oligozoospermia. In line with this study, anomalous protein content in sperm nuclei was also reported by Chevaillier *et al*. (1987) . However, both of these earlier studies failed to identify or quantify the specific content of protamine in sperm samples and the corresponding P1:P2 ratio.(Silvestroni *et al*., 1976; Chevaillier *et al.,* 1987). Balhorn *et al*. (1988) were the first to report P1:P2 ratios in sperm from a group of fertile and infertile men. Findings from this particular study clearly demonstrated an increased P1:P2 ratio in infertile men (1.58 ± 0.24) as compared to fertile men (0.98 ± 0.12) (Balhorn *et al.*, 1988). Subsequently, another study reported altered protamine levels in patients with abnormal semen parameters in comparison to patients with normal parameters, indicating that abnormal semen parameters were associated with abnormalities in protamine content (Bach *et al.,* 1990). Furthermore, round-headed sperm exhibiting reduced levels of protamine have also been reported, suggesting a link between morphologically abnormal sperm and protamine deficiency (Blanchard *et al.,* 1990).

Collectively, these early findings clearly demonstrated the significance of protamine content and P1:P2 ratios with male infertility via effects upon seminal parameters such as sperm morphology and an increase in histone content (Blanchard *et al.*, 1990). All of these previous studies above utilised acid-urea gel electrophoresis to determine protamine content.

All subsequent studies have also used the same acid-urea gel electrophoresis method and predominantly focused upon the evaluation of P1 and P2 levels, and P1:P2 ratios in fertile males in comparison to infertile males. Collectively, these studies revealed large variation of P1:P2 ratio in both fertile and infertile men, and P1:P2 ratio in human sperm was reported to be approximately 1:1 or ranging between 0.8 and 1.2 (Nanassy *et al.*, 2011). While many studies have focused upon protamine quantification in sperm using acid-urea gel electrophoresis, one particular study utilised an immunofluorescent method which successfully identified an increase in the levels of histones in sperm with protamine deficiency from infertile men (Zhang *et al.*, 2006). Subsequently, Aoki *et al*. (2006a) were the first group to evaluate P1 and P2 levels and P1:P2 ratio using immunofluorescence alongside a TUNEL assay in order to determine DNA integrity in individual sperm cells from fertile and infertile men (Aoki *et al.*, 2006a). Besides indicating variation in the P1:P2 ratio in sperm between different patients, results from this study also claimed that there were no significant differences between the two techniques (gel electrophoresis and immunofluorescent) used to quantify P1:P2 ratio. Thus far, only Zhang *et al*. (2006) and Aoki *et al*. (2006a) have attempted to evaluate protamine deficiency using immunocytochemistry. Aoki *et al*. (2006a) used both immunocytochemistry and acid-urea gel electrophoresis method to evaluate protamine content and ratio, and to compare the efficiency of both methods. In addition, immunocytochemistry was performed to evaluate protamine levels and DNA fragmentation levels simultaneously in human sperm. However, a limitation to the study reported by Zhang *et al*. (2006) was that immunofluorescent investigations were only

targeting histones and P1, not P2 (Zhang *et al.*, 2006). Furthermore, the co-localisation of histones and P1 in sperm were analysed in a random manner based upon the strength of the signal in the sperm analysed but failed to quantify the total levels of histone or P1. Furthermore, although Aoki *et al*. (2006a) demonstrated immunofluorescent quantification of P1, P2 and P1:P2 ratio in sperm cells individually from fertile and infertile men, this study did not advocate the clinical utilisation of such techniques. Furthermore, the TUNEL-assay and (TNP1 and TNP2 proteins involved in the sequential process of protamine displacement during spermiogenesis) immunofluorescence analysis were performed simultaneously, which may have interfered with the evaluation of P1 or P2 alone (Aoki *et al.*, 2006a). This, therefore, led us to further expand the use of immunocytochemistry to evaluate protamine levels for clinical application as presented in Chapter 2.

In the present study, and in a manner similar to Aoki *et al*. (2006a), I attempted to evaluate immunofluorescence levels of P1 and P2, and determine P1:P2 ratios in mouse sperm, as described in Chapter 2. The novelty of the experiments described in Chapter 2 was to demonstrate the effect of cryopreservation upon the total levels of protamine and P1:P2 ratio, and also to analyse whether the protamine immunofluorescence method might represent a novel biological assay for clinical application. Immunofluorescent methodology could represent a highly useful clinical tool since it allows the visualisation and quantification of protamine levels and localisation patterns in individual sperm, and is therefore more efficient, robust and cost effective than earlier methods. However, considering the significance of the P1:P2 ratio as a marker of good quality sperm with optimal DNA integrity, we considered it valuable to also use established acid-urea gel electrophoresis methods to investigate the effect of cryopreservation upon the total levels of P1, P2 and P1:P2 ratio in mouse sperm. Since I already determined P1:P2 ratios in non-cryopreserved and cryopreserved sperm in Chapter 2 using fluorescent immunocytochemistry, I considered that it was crucial to also evaluate

P1:P2 ratios using the established electrophoresis method in order to determine the influence of such methodology upon the accuracy and consistency of results prior to developing a clinical assay. The objective of this chapter was therefore to identify the significance, advantages and disadvantages of both protamine quantification methods, and thereby indicate the most appropriate biological tool for clinical application and the assessment of sperm DNA integrity and prediction of fertilisation outcome.

3.1.1 Specific aims of this chapter

1. To quantify the total levels of P1 and P2, and the P1:P2 ratio, in mouse sperm, by sperm protein extraction and established acid-urea gel electrophoresis methodology.
2. To investigate the effect of cryopreservation upon the total levels of protamine and P1:P2 ratio in mouse sperm in comparison to non-cryopreserved sperm using acid-urea gel electrophoresis methodology.
3. To assess and compare the significance and efficiency of fluorescent immunocytochemistry and the acid-urea gel electrophoresis method to quantify protamine levels and P1:P2 ratio in mouse sperm.
4. To attempt to identify the most suitable method for protamine quantification for potential use as a biological tool for clinical application in order to evaluate sperm DNA integrity.

3.2 Materials and Methods

3.2.1 Mouse supply and sperm preparation

Twenty-four mice, aged 16 weeks (strain: C57BL/6) were obtained and caudal sperm retrieved as described in Chapter 2 (**Section 2.2.1**). Mice were then sub-divided into four batches (Batch # 1, Batch # 2, Batch # 3 and Batch # 4), with each batch containing six mice. Sperm nuclear protein extracted from Batch # 2 was selected in random manner without any specification to prepare a series of protamine standard regression lines, and the remaining protein extract from this batch was used as a loading control. Prior to sperm nuclear protein extraction, the basic sperm parameters of the pool of caudal sperm from each batch were assessed using CASA as described in Chapter 2 (**Section 2.2.1**).

3.2.2 Mouse sperm cryopreservation and thawing procedure

Cryopreservation and thawing of pooled caudal sperm from each batch of mice was performed as described in Chapter 2 (**Section 2.2.4**).

3.2.3 Mouse sperm thawing procedure

Cryopreservation and thawing of pooled caudal sperm from each batch of mice was performed and described in Chapter 2 (**Section 2.2.4**).

3.2.4 Sperm nuclear protein extraction and acid-urea gel electrophoresis

3.2.4.1 Reagent preparation for sperm nuclear protein extraction

The reagents and buffers at the specific concentrations required for sperm nuclear protein extraction are provided in **Table 3.1**. The sperm extraction procedure was performed

as previously described by Yebra and Oliva. (1993) and Liu *et al*. (2013) and the detailed protocol is provided in **Section 3.2.4.2**.

Table 3.1: Reagents and buffers required for sperm nuclear protein extraction.

Reagents/Buffers	Final concentration	Supplier
(A) Phenylmethylsulfonyl fluoride (PMSF)	1.0 mM	Sigma Aldrich (UK)
(B) * Ethylenediaminetetraacetic acid (EDTA) Tris-hydrochloric acid (Tris-HCL) PMSF	20.0 mM 100.0 mM 1.0 mM	Sigma Aldrich (UK)
(C) * Guanidine hydrochloride Dithiothreitol (DTT)	6.0 M 575.0 mM	Sigma Aldrich (UK)
(D) Ethanol	100%	Sigma Aldrich (UK)
(E) Hydrochloric acid (HCL)	0.5 M	Sigma Aldrich (UK)
(F) Tri-Chloro Acetic Acid (TCA)	20%	Sigma Aldrich (UK)
(G) * β-mercaptoethanol Acetone	1% 100%	Sigma Aldrich (UK)
(H) * Urea β-mercaptoethanol Acetic acid	5.5 M 20% 5%	Sigma Aldrich (UK)

Asterisk (*) indicates buffer contains a mixture of the reagents listed separately.

3.2.4.2 Sperm nuclear protein extraction

Sperm nuclear protein extraction was performed according to a standardized protocol established by Yebra & Oliva (1993) and Liu *et al*. (2013). At least 10 - 20 x 10^6 mouse sperm/mL was retrieved by the swim-out technique and used to determine P1 and P2 levels and the P1:P2 ratio. Sperm suspension was centrifuged at 4000*g* for 5 minutes to obtain a sperm pellet which was then lysed by hypotonic shock with 100µl of 1.0 mM PMSF in water followed by centrifugation at 4000*g* for 5 minutes. The supernatant was discarded and the pellets were re-suspended in 100µl of buffer containing 20.0 mM EDTA + 100.0 mM Tris-HCL + 1.0 mM PMSF (**Buffer B, Table 3.1**). EDTA, Tris-HCL and PMSF were used to prevent interaction of metal ions, to maintain the pH level, and to inhibit proteinase activity respectively. The suspension was then mixed with a vortex mixer followed by the addition of 100µl 6.0 M guanidine hydrochloride and 575.0 mM DTT to reduce the disufide bonds and to decondense/dissolve sperm nuclear chromatin (**Buffer C, Table 3.1**). Subsequently, 200µl of water was added to the mixture, vortexed and incubated in the dark at room temperature for 10 minutes as described by Mengual *et al*. (2003) and Torregrosa *et al*. (2006). Following 10 minutes of incubation in the dark, the mixture was treated with 1mL of ice-cold 100% ethanol and incubated at -20°C for 1 minute to precipitate protein (protamine) in the suspension.

The ethanol mixture was then centrifuged at 14,000*g* for 8 minutes and the supernatant discarded carefully without disturbing the precipitated pellet. Nucleoproteins in the precipitated pellet were then extracted with 800µl of 0.5 M HCL by vortex mixing and subsequently incubated at 37°C for 10 minutes. Following centrifugation at 10,000*g* for 10 minutes, nucleoproteins in the supernatant were then transferred into a clean microcentrifuge tube. The extracted nucleoproteins present in the supernatant were then retrieved by precipitating with 200µl of 100% TCA followed by centrifugation at 14,000*g* for 10 minutes.

The supernatant was discarded and sperm nucleoprotein was collected under reducing condition by adding 1mL of 1% β-mercaptoethanol in 100% acetone (**Buffer G, Table 3.1**) with centrifugation at 14,000*g* for 10 minutes. Following the complete removal of supernatant, nucleoprotein pellets were allowed to air dry for 20 minutes in a fume hood, and then dissolved in 20µl or a required volume up to 100µl of solution containing 5.5 M urea + 20% β-mercaptoethanol + 5% acetic acid (**Buffer H, Table 3.1**). Mouse sperm nucleoproteins were then ready to be loaded onto an acid-urea gel for electrophoresis.

3.2.4.3 Reagents required for acid-urea gel electrophoresis

The reagents and buffers at their required concentration for the preparation of acid-urea running gel electrophoresis and protamine separation from extracted sperm nuclear proteins are given in **Table 3.2**.

Table 3.2: Reagents and buffers required to prepare acid-urea running gel for electrophoresis.

Reagents	Volume	Supplier
40% Acrylamide/Bis solution 19:1	8.0mL	Biorad (UK)
6.67M Urea	6.0mL	Sigma Aldrich (UK)
1.6% Ammonium persulfate	2.0mL	Sigma Aldrich (UK)
43.1% of Acetic acid	2.0mL	Sigma Aldrich (UK)
Tetramethylethylenediamine (TEMED)	70.0µL	Sigma Aldrich (UK)
* Sample loading buffer: 0.75M Potassium acetate, pH 4.0 30% Sucrose 0.1% Pyronin Y	#Required volume	Sigma Aldrich (UK)
* Coomassie blue solution: 2.5g Brilliant blue R250 10% Acetic acid 30% Methanol	10.0mL	Sigma Aldrich (UK)
* De-staining solution: 10% Acetic acid 30% Methanol	10.0mL	Sigma Aldrich (UK)
Asterisk (*) indicates buffer contains a mixture of the reagents listed separately. Hash mark (#) indicates sample loading buffer was added in volume equal to that sperm nuclear extract to be loaded onto the gel.		

The acid-urea separation gel was prepared as described by Liu *et al*. (2013) using the reagents listed in **Table 3.2**. Approximately 8.0mL of 40% Acrylamide/Bis solution was mixed with 6.0mL of 6.67M urea. Then, 2.0mL of 1.6% AP in 43.1% acetic acid was added to the mixture. Finally, 70µl of TEMED was added and the gel was poured into a 1.0 mm thick gel cassette followed by insertion of a cassette comb (Life Technologies, UK). The gel was then allowed to solidify for an hour.

3.2.4.4 Separation of P1 and P2 proteins by acid-urea gel electrophoresis

Mouse sperm extracts were separated by acid-urea gel electrophoresis as described by Yebra & Oliva, (1993), Carrel & Liu (2001) and Liu *et al*. (2013). Approximately 10µl of extracted mouse sperm nuclear protein was mixed with an equal volume of sample loading buffer (**Table 3.2**) and briefly centrifuged. The acid-urea gel prepared as described in **Section 3.2.4.3** was placed in an electrophoretic chamber and filled with 500mL of 0.9N acetic acid running buffer. First, the gel was pre-run at 150 Volt (V) and 30 milliamps (mA) for 2 hours to remove ammonium persulfate from the gel. Ammonium persulfate contains charged ions that interfere with positively-charged protamine molecules. Such interference would affect the separation of protamine in sperm nuclear extracts. Therefore, the pre-run step was an essential step prior to sample loading and separation. A total volume of 20µl per sample (10µl of sperm nuclear protein extract + 10µl of sample loading buffer) was then loaded into the gel wells and electrophoretic separation performed at 150 Volts (V) and 300 milliamps (mA) for 3 hours and 40 minutes. Following electrophoresis, the gel was stained for at least 1 hour with coomassie blue stain (**Table 3.2**). Subsequently, the gel was de-stained overnight with a solution consisting of 10% acetic acid + 30% methanol. Finally, the wet gel was scanned using a UVP GelDoc-It imaging system (Upland, CA, USA). The relative densities of resultant P1 and P2 bands were measured using Quantity One software version 4.6.2 (Bio-

Rad, Richmond, CA, USA). Briefly, bands were selected and the outline of bands was first used to evaluate the background intensity of the gel. Thereafter, the protein band intensity was measured using the same outline. The density of the protein was then evaluated by subtracting the background intensity with the protein intensity. Data are given in arbitrary units (AU). Following this, the P1:P2 ratio was calculated by dividing the densities of P1 bands by the densities of P2 bands. The relative densities of P1 and P2 protein present in discrete bands resulting from the total amount of protein loaded were measured by correlating the densities of each P1 and P2 band with protamine standard regression model. Preparation of protamine standard regression line and the relevant data are described and presented in **Section 3.3.1**.

3.2.5 Immunoblotting for the identification of P1 and P2

Separated mouse P1 and P2 bands in acid-urea gels were further identified by Western Blotting (Hammadah *et al*., 2010) using two monoclonal protamine antibodies, Hup1N and Hup2B (Briar-Patch, LLC, USA), specific to P1 and P2 respectively. Mouse sperm nuclear protein extracts were loaded in duplicates into gel wells and separated by electrophoresis as described in **Section 3.2.4.4**. An increasing series of nuclear protein extract was loaded (7.5µg, 10µg and 12.5µg). Each amount was loaded in duplicate to create two replicates. Two replicates were generated to treat the protein bands transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham GE Healthcare, UK) with P1 and P2 antibodies separately for each replicate. Following electrophoresis, gels were transferred to the PVDF membrane using a wet transfer blotting system, X-Cell II Blot Module (Life technologies, UK) in 0.0009N acetic acid at 300 mA and 75V for 2 hours. Following protein transfer, the PVDF membrane was divided and cut into two separate membranes consisting of the two replicates as shown in **Figure 3.1**. Each membrane was blocked with 5% skimmed

milk solution in 0.1% Tween-20 (Sigma Aldrich, UK) for 1 hour. Following a blocking step, each membrane was incubated overnight at 4°C separately with Hup1N and Hup2B antibodies (**Figure 3.1**) diluted by 1:2000 to obtain a final concentration of 0.5µg/mL in 1% skimmed milk. Next, membranes were incubated for an hour at room temperature with horseradish peroxidase (HRP) conjugated goat-anti mouse IgG secondary antibody (Insight Biotechnology, UK) diluted by 1:10,000 to obtain a final concentration of 0.02µg/mL. Membranes were then incubated with Western Blot detection reagent, enhanced chemiluminescence (ECL) prime (Amersham, GE Healthcare, UK) for 1-3 minutes in order to visualize P1 and P2 bands.

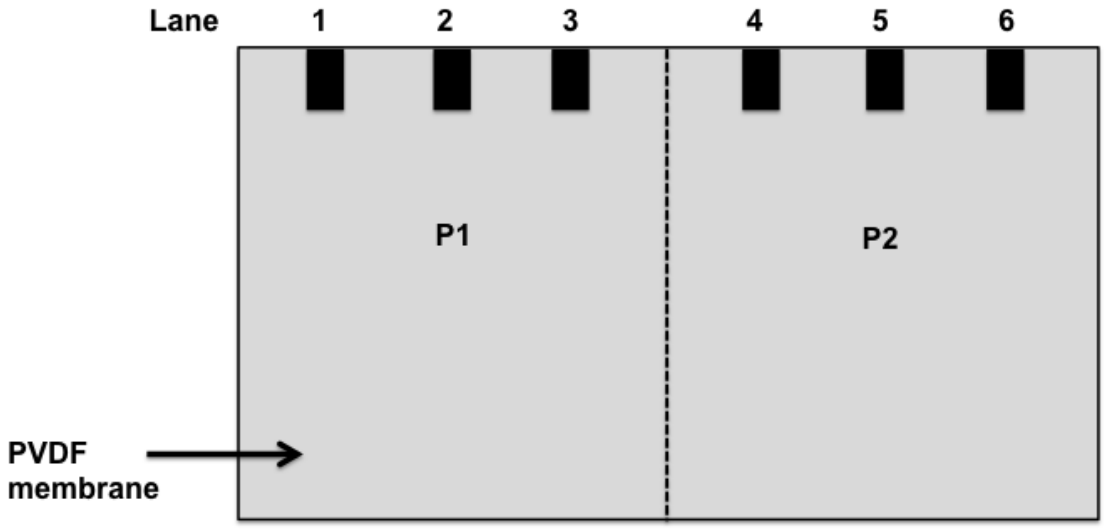


Figure 3.1: Polyvinylidene fluoride (PVDF) membrane divided into two sections following protein transfer. Lane 1-3, Lane 4-6, represent an increasing amount of mouse nuclear protein extracts (7.5µg, 10µg, 12.5µg) loaded in duplicates. The first part of the membrane was incubated with Hup1N antibody specific to protamine 1 (P1) whereas the second part was incubated with Hup2B antibody specific to protamine 2 (P2).

3.2.6 Determination of P1 and P2 band identity by liquid chromatography and mass spectrometry analysis (LC/MS)

An aliquot of mouse sperm nuclear protein extract was loaded into an acid-urea gel and protamine bands separated by electrophoresis as described in **Section 3.2.4.4**. Following electrophoresis, the slower migrating band (P2) and the faster migrating band (P1) were excised and stored in separate 1.5ml tubes containing acetic acid. The samples were then sent to Prof. Benedikt M Kessler and Dr. Roman Fischer, Target Discovery Institute (TDI), Nuffield Department of Medicine, University of Oxford for LC/MS analysis. Upon delivery, the samples were cut into smaller pieces and washed in buffer containing methanol and acetic acid. Then, the proteins in the gel pieces were cleaved into shorter peptides using 'Elastase'. This enzyme was used due to its ability to cause cleavage at any residue, in contrast to the usually employed enzyme Trypsin, which is specific to arginine and lysine residues. The frequency of Arginine in P1 as well as in P2 would otherwise have prevented the generation of mass spectrometry (MS)-detectable protein fragments. Peptides were extracted using buffer containing acetonitrile and formic acid. Samples were then centrifuged to collect the supernatant, followed by vacuum centrifugation and re-suspended in 20µl of buffer containing 1% acetonitrile and 0.1% formic acid. In order to finally identify the peptides corresponding to mouse P1 and P2, the final suspension of sample was first separated using High Performance Liquid Chromatography (HPLC, Dionex Ultimate 3000). The separated and ionized peptides were then transferred into a Mass Spectrometer (Thermo Q-Exactive MS) by electrospray (ESI) whereby the peptide precursor masses and their fragmentation products were detected in an orbitrap. Finally, all spectra generated were compared and matched to theoretical peptide sequences generated from the annotated mouse proteome deposited at Uniprot, www.uniprot.org. The accession numbers for mouse P1 and P2 were SP (P02319) HSP1_MOUSE and SP (P07978) PRM2_MOUSE.

3.2.7 Statistical analysis

Data obtained from the 18 mice were expressed as mean ± SEM and statistically analysed using IBM SPSS 20.0 for Windows (IBM Corp.; Chicago, Illinois). All data were first analysed using the Shapiro-Wilks test to check for a normal distribution across the numerical values. Secondly, homogeneity of variance (homoscedasticity) was assessed using the Levene's test. Effects of cryopreservation on the total levels of P1, P2, and upon P1:P2 ratios were evaluated by a general linear model, where the treatment (non-cryopreserved vs. cryopreserved) was a fixed-effect factor and the batches of mice was a random-effect factor. Dependent variables were the total levels of P1, total levels of P2 and the P1:P2 ratios. Significance level was set at $P \leq 0.05$.

3.3 Results

3.3.1 Protamine standard regression lines

Protamine standard regression lines were generated using sperm nuclear protein extracted from the pool of caudal sperm from the six mice in Batch # 2. Prior to sperm nuclear protein extraction, sperm parameters were assessed using CASA as described in Chapter 2 (**Section 2.2.1**). The total concentration of sperm obtained from Batch # 2 was 6.8 M/mL. The final pellet obtained from sperm nuclear protein extraction was dissolved in 100µl of solution containing 1% β-mercaptoethanol in 5% acetic acid. The final protein concentration of the 100µl sperm nuclear protein extract containing purified protamine was determined using a reducing agent and detergent compatibility (RC-DC) protein assay kit (Bio-Rad, UK) as described by Hammadah *et al.* (2011). Subsequently, the final concentration of the sperm nuclear protein extract obtained was 2.5µg/µl. A known concentration of the sperm nuclear protein extract, or serial dilutions to prepare protamine

standards (5µg, 10µg, 15µg, and 25µg), were then loaded and separated by acid-urea gel electrophoresis (**Figure 3.2**) as described in **Section 3.2.4.4**. The protamine standard regression lines (**Figure 3.3**) were formulated based upon density measurement of P1 and P2 bands corresponding to each sample concentration loaded in the gel. Increasing densities of each P1 and P2 bands obtained corresponded accurately to the increasing amount of mouse sperm nuclear protein extract loaded and separated in the gel. R^2 values of the regression models for P1 and P2 were lower than 0.99. Protein ladders (to determine molecular mass of target proteins) and loading controls (to confirm equal loading of sample for Western Blot) used in common electrophoresis and Western blot analysis are not applicable for acid-urea gel electrophoresis analysis. This is because, the acid-urea gel electrophoresis method is specialised to separate highly basic proteins in a reducing condition. The protocol used and described in **Section 3.2.4.2** was specifically design to extract sperm nuclear proteins, which contains only P1 and P2, and were separated using acid-urea gel electrophoresis. The P1 and P2 bands were thus identified based upon the size, molecular weight and charge of these proteins. The protamine standard regression lines were used to determine concentration of the proteins and to ensure equal loading of sperm nuclear protein extracts.

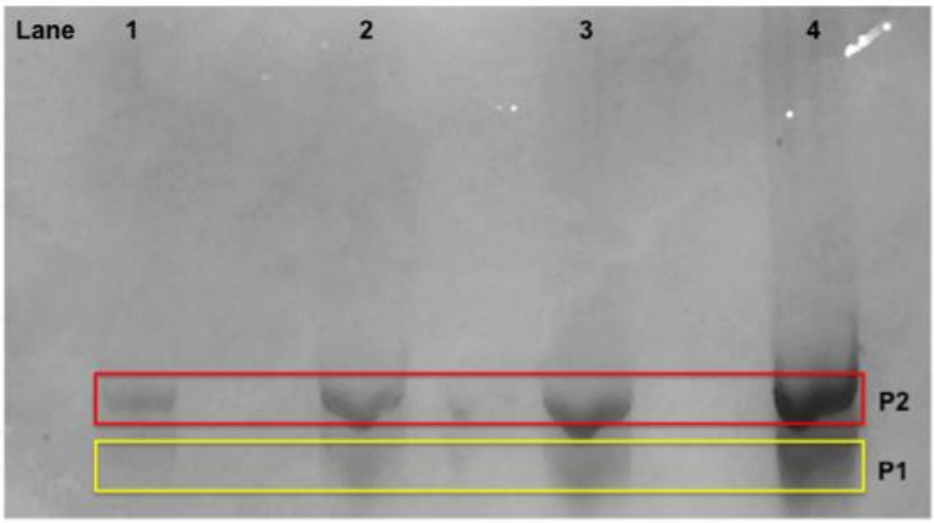


Figure 3.2: Nuclear protein extracted from a pool of mouse caudal separated in acid-urea gel stained with Coomassie Blue. Lane 1 – 4 corresponds to an increasing concentration of nuclear proteins. Red and yellow rectangular box indicates slower migrating bands (P2) and faster migrating bands (P1) respectively.

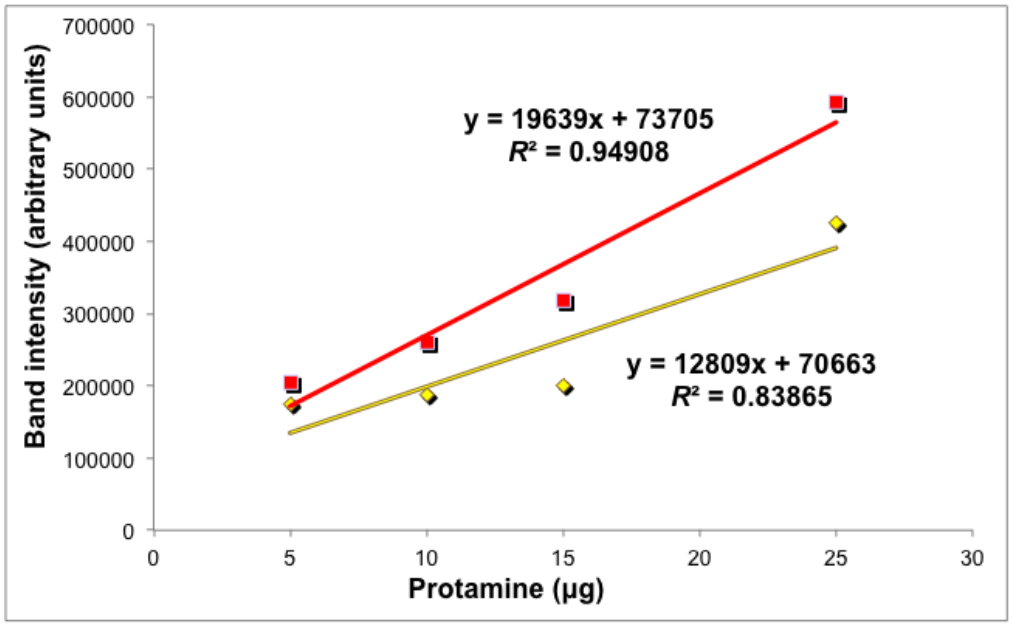


Figure 3.3: Standard regression model for protamine. Red line represents P2 and yellow line represents P1.

3.3.2 Evaluation of total levels of P1, P2, and P1:P2 ratio, in non-cryopreserved and cryopreserved mouse sperm by acid-urea gel electrophoresis

CASA results for all three batches of non-cryopreserved and cryopreserved mouse sperm are presented in **Table 3.3**. The mean sperm parameters for each batch of mice, total motility, progressive motility and the concentration of cryopreserved sperm were significantly reduced compared to non-cryopreserved ($P \leq 0.05$; as indicated by the Student's *t*-test).

Table 3.3: Mean computer assisted sperm analysis (CASA) data of pooled caudal mouse sperm from three batches of mice prior to and following cryopreservation.

Total number of batches (n=3)	Sperm assessment		
	Non-cryopreserved sperm		
	Total sperm motility	Sperm progressive motility	Sperm concentration (M/mL)
Mean ± SEM	84.0 ± 5.6	23.3 ± 4.4	11.3 ± 0.1
	Cryopreserved sperm		
	Total motility	Sperm progressive motility	Sperm concentration (M/mL)
Mean ± SEM	0.7 ± 0.6*	0.0*	7.4 ± 0.3*

Asterisks (*) denote statistically significant differences ($P \leq 0.05$) in total sperm motility, sperm progressive motility and sperm concentration between non-cryopreserved and cryopreserved mouse sperm**.** Data are shown as mean ± SEM.

Total levels of P1 and P2 in non-cryopreserved and cryopreserved sperm from each batch were analysed in three replicates by acid-urea gel electrophoresis and coomassie staining (**Figure 3.4**) was quantified by densitometry using Quantity One software version 4.6.2 (Bio-Rad, Richmond, CA, USA). Mean P1:P2 ratios from the three replicates of each batch of non-cryopreserved and cryopreserved sperm were evaluated by dividing the mean band intensities of P1 by the mean intensities of P2.

Data obtained are shown in **Table 3.4**. Statistical analysis revealed that the mean total levels of P1 and P2 in cryopreserved sperm from all three batches were significantly reduced by 38% and 37% respectively, as compared to non-cryopreserved sperm. Considering the total levels of P1 as a dependent variable, the total levels of P1 showed significant variability

between each batch of sample and in comparison to the effect of cryopreservation. Similar significant variability was observed in the total levels of P2 between each batch of sample and the effect cryopreservation. However, the mean P1:P2 ratio evaluation did not reveal any statistical significant differences between non-cryopreserved 0.71 and cryopreserved sperm 0.71 from the three batches when analysed collectively. Moreover, the P1:P2 ratio between each batch of mice and in comparison to the effect of cryopreservation did not show any statistically significant differences. The standard regression lines for P1 and P2 were not used to evaluate the concentration of P1 and P2 because the R^2 value was lower than 0.99 which may indicate inaccuracy in concentrations of P1 and P2.

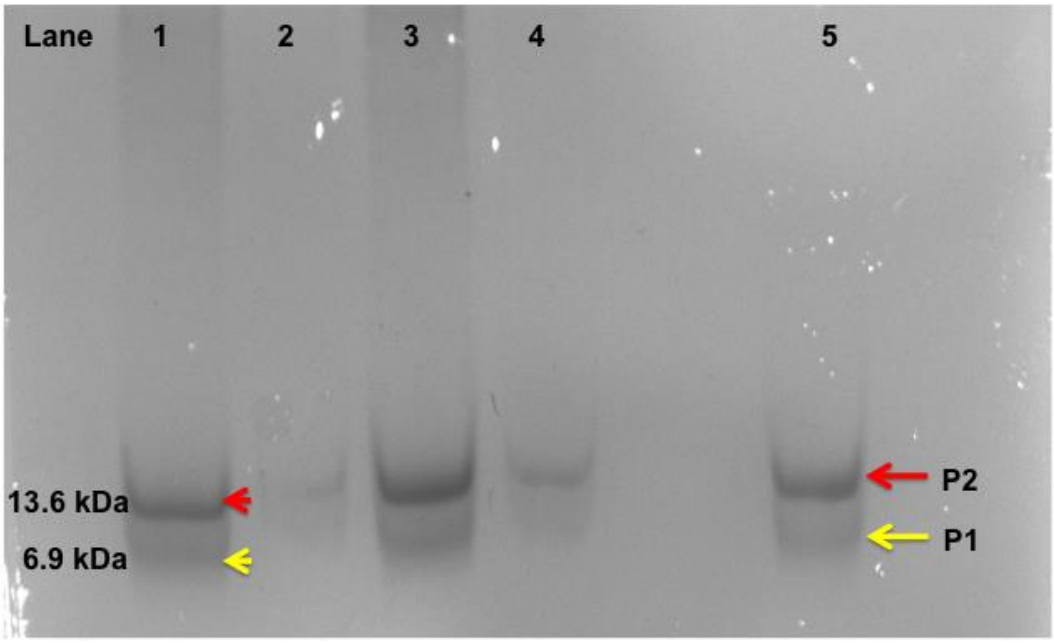


Figure 3.4: Nuclear protein extracted from a pool of non-cryopreserved and cryopreserved mouse caudal sperm in two replicates per batch separated in acid-urea gel and stained with coomassie blue. Lanes 1 and 3 correspond to an unknown amount of nuclear protein extracted from non-cryopreserved sperm whereas lane 2 and 4 represents nuclear protein extracted from cryopreserved sperm. Lane 5 corresponds to 12.5µg of protamine standard which was used as a control sample. Red and yellow arrow indicates slower migrating band P2 and faster migrating band P1 respectively. Red and yellow arrow head indicated the molecular weight of P2 and P1 respectively.

Table 3.4: Mean densitometry value of P1, P2, and P1:P2 ratio for non-cryopreserved and cryopreserved sperm from all three batches of mice.

Batches (n=3)	P1	P1-cryo	P2	P2-cryo	P1:P2	P1:P2 cryo
Mean ± SEM	* 294057 ± 72253	* 181467 ± 66625	** 406583 ± 79201	** 254149 ± 93909	0.71 ± 0.04	0.71 ± 0.03

Asterisks (*, **) denote statistically significant differences ($P \leq 0.05$) in the total levels of protamine 1 (P1) and protamine 2 (P2) respectively between non-cryopreserved and cryopreserved sperm. Band intensities were quantified in arbitrary units (AU).
Data are shown as mean ± SEM.

3.3.3 Determination of P1 and P2 band identity

3.3.3.1 Liquid chromatography and mass spectrometry (LC/MS)

The P1 and P2 bands separated by acid-urea gel electrophoresis were excised and the identity of these bands determined using a proteomic protocol. It was important to confirm that the sperm nuclear protein extraction contained P1 and P2, and that the bands being quantified were indeed the P1 and P2 target proteins. Acid-urea gel electrophoresis successfully separated two bands of the appropriate molecular weight. However, LC/MS was the only way to confirm the identity of these two bands by analysing the protein sequence in each discrete band from the gel. The excised P1 and P2 gel bands (**Figure 3.5B**) were sent to the LC/MS service (Target Discovery Institute, Nuffield Department of Medicine, University Oxford). Subsequently, the amino acid sequences of the protein bands corresponding to mouse P1 and P2 amino acid sequences were clearly identified (**Figure 3.5A**). The discovery rate obtained for P1 and P2 was 86.5% and 83.3% respectively, which confirmed successful identification.

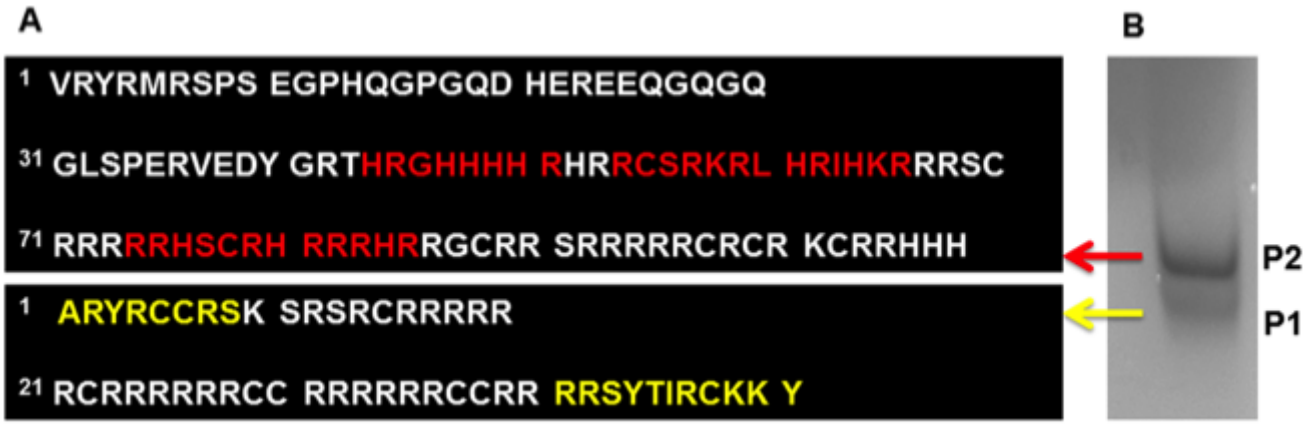


Figure 3.5: The complete amino acid sequence of mouse protamine 1 (P1) and (P2) 2. (**A**) Amino acid sequences identified from the bands (**B**) excised from an acid-urea gel. Red font represents the amino acid for P2 whereas the yellow font shows the amino acids for P1 recognised by LC/MS. Red and yellow arrow indicates slower migrating band (P2) and faster migrating band (P1) respectively.

Figure 3.5A (upper panel) shows the complete amino acid sequence of mouse P2. Highlighted in red font are those successfully identified amino acids in the excised slower migrating band from the gel segment (as indicated by red arrow in **Figure 3.5B**) sent for LC/MS. Similarly, the lower panel of **Figure 3.5A** shows the amino acid sequence for mouse P1 and the yellow font shows those amino acids recognised in the faster migrating band excised from the gel segment (as indicated by yellow arrow in **Figure 3.5B**) and sent for LC/MS.

3.3.3.2 Validating the identity of P1 and P2 by immunoblotting

In addition to the formal identification of P1 and P2 by LC/MS as presented in **Section 3.3.3.1**, an in-house validation was also performed using immunoblotting. Following acid-urea gel electrophoresis, immunoblotting was performed on the unstained gels using two monoclonal protamine antibodies (Hup1N and Hup2B) to identify P1 and P2 bands in sperm nuclear protein extracts as described in **Section 3.2.5**. Western blot results obtained from two gel replicates are shown in **Figure 3.6**.

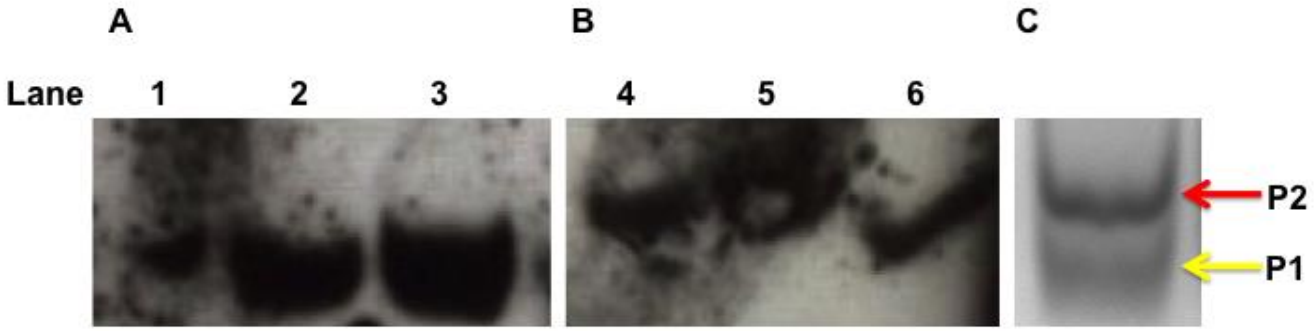


Figure 3.6: Identification of protamine 1 (P1) and protamine 2 (P2) in acid-urea gels using immunoblotting technique. (**A**)Western blot corresponding to the example of coomassie-stained gel shown in C using Hup1N antibody, specific to protamine 1 (P1). (**B**) Western blot corresponding to the example of coomassie-stained gel shown in (**C**) using Hup2B antibody, specific to protamine 2 (P2). Lane 1-3, Lane 4-6, represent increasing amount of mouse sperm nuclear extract (7.5µg, 10µg, 12.5µg) loaded into each lane. Red and yellow arrow indicates the slower migrating P2 band and the faster migrating P1 band respectively.

An increasing amount of sperm nuclear protein extract (7.5µg, 10µg, 12.5µg) was loaded onto gels and corresponding P1 (**Figure 3.6A**) and P2 (**Figure 3.6B**) bands were successfully identified following Western blot analysis. This experiment thus provided additional evidence to support the fact that target bands of interest and being quantified were indeed P1 and P2 and therefore did not represent non-specific binding of unrelated proteins.

3.4 Summary of results

1. Protamine standard regression lines were successfully generated.
2. Total levels of P1 and P2 were significantly reduced in cryopreserved mouse sperm compared to non-cryopreserved sperm as determined by acid-urea gel electrophoresis.
3. P1:P2 ratio remained unchanged following cryopreservation and likely to be attributed to similar levels of reduction in P1 and P2 following cryopreservation.
4. P1:P2 ratio in mouse sperm determined by the acid-urea gel electrophoresis and densitometry analysis may represent a better method to evaluate P1:P2 ratio in mouse sperm compared to the immunofluorescent method as described in Chapter 2.

3.5 Discussion

The results described in this chapter represent the first study to investigate the effect of cryopreservation upon the total levels of P1, P2 and P1:P2 ratio in cryopreserved mouse sperm in comparison to non-cryopreserved sperm by direct assessment using an acid-urea gel electrophoresis method.

Previous studies have demonstrated abnormalities in protamine content and P1:P2 ratio in sperm from infertile males as compared to fertile males. Collective findings from these previous studies showed high variability in the P1:P2 ratio obtained (Oliva, 2006; Nanassy *et al.*, 2011) using acid-urea gel electrophoresis. Similarly, the content of P1 and P2 among different men, either fertile controls or infertile patients, also varies significantly in the published literature (Nanassy *et al.*, 2011) (**Table 3.6**). In line with these studies, several other reports demonstrated the association of abnormal semen parameters with increased DNA fragmentation levels and abnormal P1:P2 ratios in infertile men (Aoki *et al.*, 2005a; Aoki *et al.*, 2006a; Simon *et al.*, 2010; Castillo *et al.*, 2011). Such findings indicate the vulnerability of sperm to DNA damage in relate to abnormal protamination (Aoki *et al.*, 2006a; Castillo *et al.*, 2011) and thus suggest that the analysis of protamine in clinical samples is important. Protamine ratio in normal human sperm has been postulated to be approximately 1:1 or fall within a narrow range of 0.8-1.2 (de Mateo *et al.*, 2009). However, recent reports have collectively suggested that protamine ratio ranges more between 0.53-1.43 in fertile men (Nanassy *et al.*, 2011). **Table 3.5** provides a summary of previous literature, which clearly shown variation in P1:P2 ratio. Since many authors appear to advocate the use of protamine analysis as a clinical scale, the variation evident in published literature (Nanassy *et al.*, 2011) is worrying and implies potential influence of the acid-urea gel electrophoresis methodology used.

Table 3.5: Mean P1:P2 ratio and range of ratios in the published literature.

Studies	Group	N	P1:P2 ratio	Range	Sperm count
Balhorn *et al.* (1988)	Fertile	17	0.98 ± 0.12	0.79-1.27	N/A
Belokopytova *et al.* (1993)	Fertile	20	0.99 ± 0.06	0.87-1.11	N/A
Khara *et al.* (1997)	FR > 50%	12	0.84	0.55-1.29	(8 –14) x 10^6
Carrell and Liu (2001)	Fertile	50	0.83 ± 0.05	0.53-0.99	N/A
Mengual *et al.* (2003)	Fertile	10	1.01 ± 0.15	0.68-1.43	>20 x 10^6
Aoki *et al.* (2005b)	Fertile	87	1.06 ± 0.01	0.75-1.26	N/A
Mateo *et al.* (2009)	Fertile	12	1.30 ± 0.10	1.10-1.40	14 x 10^6
		208	**0.99**	**0.53-1.43**	

Data adapted and modified from Nanassy *et al.* (2011).
N= Number of fertile controls.
FR= Fertilisation rates.
N/A= Data not available.
Data are shown as mean, mean ± SD, or mean ± SEM in accordance with study concerned.

The results of Chapter 2 used quantitative immunofluorescence to demonstrate significantly reduced levels of P1 and P2 in cryopreserved mouse sperm as compared to non-cryopreserved sperm. This indicated that cryopreservation exerts a significant effect over the total levels of protamine and may therefore compromise DNA integrity of the affected sperm. The P1:P2 ratio obtained in non-cryopreserved sperm was 1.0 as compared to 1.1 in cryopreserved sperm. The immunofluorescent method was used in Chapter 2 because, firstly, no studies have used this approach, especially to determine protamine levels in cryopreserved sperm. Secondly, this method allowed both the quantification of protamine levels and

visualisation of localisation patterning within a sperm efficiently. Thus far, there have been no investigations of protamine quantification in mouse sperm either by acid-urea gel electrophoresis or immunocytochemistry to indicate fertility disorders and to study the effect of iatrogenic damage upon protamine. However, evidence is mounting to indicate the link between protamine abnormalities and P1:P2 ratio and fertility disorders in humans (Francis *et al.*, 2014).

Previously protamine evaluation in mouse sperm predominantly aimed to determine P1:P2 ratio and the amino acid sequences of P1 and P2 (Balhorn *et al.*, 1977; Corzett *et al.*, 2002). Several other studies aimed to generate P1 and P2 knock-out mice to demonstrate the significance of protamine insufficiency and links to infertility, DNA fragmentation and embryonic death (Cho *et al.*, 2001; Cho *et al.*, 2003). Corzett *et al*. (2002) reported P1:P2 ratio in mouse sperm to be 1:2 evaluated based on gel electrophoresis and HPLC (Corzett *et al.*, 2002). However, as indicated in Chapter 2, our P1:P2 ratio obtained via an immunocytochemistry method ranged from 0.99 to 1.13. It is thus apparent that the choice of method appears to influence the results obtained, and that such factors used to be taken into account when considering clinical application.

The work in this chapter therefore performed to determine the levels of P1, P2 and the P1:P2 ratio using acid-urea gel electrophoresis. In Chapter 2, we predominantly focused upon investigating the effect of cryopreservation upon total levels of protamine and to develop a novel immunofluorescent protamine assessment assay. This experimental approach was essential, firstly to determine the significance of iatrogenic damage upon protamine. Secondly, to assess whether the immunofluorescent assay may be an efficient tool compared to acid-urea gel electrophoresis for clinical assessment to determine sperm fertilisation ability. Our immunofluorescent method resulted in a P1:P2 ratio that differed from earlier publications. This prompted us to compare findings from quantitative fluorescent

immunocytochemistry with established acid-urea gel electrophoresis, to investigate whether the choice of technique can influence results. This chapter therefore had two clear aims, to firstly investigate if P1:P2 ratio obtained via acid-urea gel electrophoresis would yield a P1:P2 ratio that was more in line with published data. Secondly, to determine if the effect of cryopreservation upon total levels of protamine in mouse sperm was similar as those seen in Chapter 2. Data arising may therefore allow us to more accurately determine if immunofluorescent methods could represent a potential tool for protamine assessment in clinic.

Results first revealed the significant variability of P1 and P2 content between the three batches of mouse sperm. Furthermore, and in line with Chapter 2, the total levels of P1 and P2 in cryopreserved mouse sperm were significantly reduced as compared to non-cryopreserved sperm. These findings indicate that the two methods used herein (immunocytochemistry and acid-urea gel electrophoresis) to evaluate total levels of protamine in mouse sperm were indeed comparable. Similar findings were previously reported by Aoki *et al*. (2006b) using human sperm using both acid-urea gel electrophoresis and immunocytochemistry.

The P1:P2 ratio obtained in this chapter was not significantly different between non-cryopreserved and cryopreserved mouse sperm, and ranged from 0.66 to 0.77 in non-cryopreserved sperm and cryopreserved sperm respectively. Interestingly, the P1:P2 ratio demonstrated in Chapter 2 following quantitative immunofluorescent yielded higher values ranging from 0.99 to 1.13. The current findings may therefore suggest that acid-urea gel electrophoresis may represent a better technique with which to evaluate P1:P2 ratio in mouse sperm at least, since the range of ratios obtained 0.66–0.77 were more in line with that described in the literature (Corzett *et al.*, 2002) and that perhaps the quantitative immunofluorescent approach provided an over-estimate.

Thus far, most studies using acid-urea gel electrophoresis for protamine quantification and P1:P2 ratio determination in human sperm have demonstrated a large variation of ratios, even in fertile men, which may suggest that the acid-urea gel electrophoresis method may not necessarily be the best assessment tool for P1:P2 ratio in human sperm or that the observed variation is real. **Table 3.6** shows a summary of published data showing a range of P1:P2 ratios arising from fertile and infertile men (Hammadeh *et al.*, 2010; Castillo *et al.*, 2011; Garcia-Peiro *et al.*, 2011; Simon *et al.*, 2011) using acid-urea gel electrophoresis. These studies show wide variation in P1:P2 ratio.

Recently, the mRNA ratio of P1:P2 was suggested as perhaps being a potential clinical parameter with which to predict fertilisation ability in men participating in ART treatment (Rogenhofer *et al.*, 2013; Ni *et al.*, 2014). This method could be a better tool compared to acid-urea gel electrophoresis, since the evaluation of protamine ratio involved investigated unexpressed protamine mRNA, which also enabled determination of potential genetic abnormalities in both fertile and infertile men. Although the utilisation of mRNA techniques may represent an advancement and a reliable source to evaluate P1:P2, current studies based on P1:P2 mRNA ratio are very limited and in terms of using such a technique for clinical application may incur high costs and would require skilled expertise in RNA protection. Interestingly, mRNA evaluation also showed variability in P1:P2 ratio among fertile males and in some cases, the range of P1:P2 ratios among infertile men were comparable to fertile men (**Table 3.6**).

Table 3.6: Mean P1:P2 ratio, P1:P2 mRNA ratio, and range of ratios from fertile, infertile and sub-fertile groups in published literature.

Studies	Group	N	P1:P2 ratio	Range	Sperm count
Hammadah *et al.* (2010)	Fertile	63	1.11 ± 0.20	N/A	(20-40) x 10^6
Garcia-Piero *et al.* (2011)	Fertile	6	1.23 ± 0.48	0.50-1.90	14 x 10^6
Castillo *et al.* (2011)	Infertile	66	0.93± 0.13	0.63-1.24	>20 x 10^6
Simon *et al.* (2011)	Infertile	97	0.90 ± 0.02	N/A	10 x 10^6
Studies	**Group**	**N**	**P1:P2 mRNA ratio**	**Range**	**Sperm concentration (M/mL)**
Rogenhofer *et al.* (2013)	Fertile	32	0.93 ± 0.30	N/A	80.45 ± 42.10
	Sub-fertile	306	0.80 ± 0.15	0.64-1.00	23.80 - 44.80
Ni *et al.* (2014)	Fertile	10	1.11 ± 0.13	N/A	33.41 ± 8.52
	Sub-fertile	42	2.11 ± 1.11	N/A	13.01 ± 6.31

N= Number of fertile controls, infertile patients and sub-fertile patients.
N/A= Data not available.
Data are shown as mean ± SD, or mean ± SEM in accordance with study concerned.

Previous studies have demonstrated that P1:P2 ratio abnormalities in human sperm, arise as either an increase or reduction in protamine content, thus leading to fertility disorders (de Yebra *et al.*, 1998; Carrell & Liu, 2001; Aoki *et al.*, 2006b; Carrell *et al.*, 2007; de Mateo *et al.*, 2009). For example, the under- or over-expression of P1 or P2 due to spematogenic or epididymal maturation defects can lead to alterations of P1:P2 ratio (Francis *et al.*, 2014). Most studies showing a high P1:P2 ratio among infertile men are frequently associated with reduced levels of P2 (Carrell *et al.*, 2007).

Considering the amino acid sequences of P1 and P2, it is clearly evident that P2 possesses fewer cysteine groups (Balhorn, 2007; Gosalvez *et al.*, 2011) which are required to form disulfide bridges. During the post-translation modification of protamine in maturing sperm, the cysteine groups in P1 and P2 are oxidized to form disulfide bonds which cross-links protamine with DNA. This mechanism would enhance protamine-DNA compaction leading to the formation of a toroidal structure (Balhorn, 2007; Carrell *et al.*, 2007). A reduced number of cysteine groups could influence the formation of disulfide cross-links leading to poor sperm DNA integrity (Gosalvez *et al.*, 2011). In mature sperm, the number of disulfide bridges formed is thought to be fewer in P2 than P1, which may increase the vulnerability of P2 to potential internal or external detrimental factors, and lead to DNA compaction problems (Aoki *et al.*, 2005a; Carrell *et al.*, 2007). As reported by Gosalvez *et al.* (2011), the resistance of sperm to cryopreservation-induced DNA damage in different species was linked to the number of cysteine groups in the P1 and P2 group of the respective species (Gosalvez *et al.*, 2011). Hence this study clearly suggested that the prevalence of cysteine in protamine plays a significant role in determining the compaction of protamine-DNA packaging in sperm and that fewer cysteine groups may lead to an increase in the susceptibility of sperm to DNA damage or abnormal protamination (Gosalvez *et al.*, 2011).

In mouse sperm, there are 9 and 7 cysteine residues for P1 and P2, respectively. As demonstrated in Chapter 2, the reduction of P2 in cryopreserved sperm was higher (51%) than P1 (45%) which may relate to the susceptibility of P2 to cryo-damage owing to a lower number of cysteine groups. However, as shown in the current chapter, the relative reduction of both P1 and P2 by 38% and 37%, respectively, in mouse sperm following cryopreservation was almost identical, strongly suggesting that different quantification techniques may influence the results obtained, especially when the acid-urea gel electrophoresis method involves extraction of nuclear protein from whole sperm, as compared to the

immunofluorescent quantification of sperm individually (Chapter 2). Alterations in P1:P2 ratio may not be applicable, or play a fundamental role in the current study design, since the reduction in P1 and P2 levels were identical and no significant alterations to P1:P2 ratio were observed between non-cryopreserved and cryopreserved mouse sperm. Instead the P1:P2 ratio was comparable prior to and following cryopreservation.

It is possible that the lower concentration of sperm in the cryopreserved sample ranging from (7.1 M/mL - 7.9 M/mL) as compared to that in the non-cryopreserved sample (10.8 M/mL – 11.8M/mL) that was used for nuclear protein extraction may have influenced levels of P1 and P2 observed in cryopreserved sperm following densitometry quantification. However, the total concentration of sperm used for this method by previous researchers varied between 5.0 M/mL and 40.0 M/mL (Carrell & Liu, 2001; Aoki *et al.*, 2005a; Torregrosa *et al.*, 2006; Hammadeh *et al.*, 2010; Castillo *et al.*, 2011; Garcia-Peiro *et al.*, 2011). These studies did not show consistency in terms of the concentration of sperm used for nuclear protein extraction. Consequently, the range observed in the present study was consistent with previous literature.

The current study encountered several drawbacks which may have restricted the determination of protamine concentration for each separate batch of nuclear protein extracts. Firstly, the concentration of P1 and P2 for each batch of mouse sperm prior to and following cryopreservation could not be evaluated robustly since the R^2 regression value obtained for both the P1 and P2 standards was below 0.99. Instead, protamine levels and ratio were determined by densitometry measurement, as used commonly in previous studies (Carrell & Liu, 2001; Mengual *et al.*, 2003; Aoki *et al.*, 2005a; Torregrosa *et al.*, 2006; de Mateo *et al.*, 2009; Hammadeh *et al.*, 2010; Garcia-Peiro *et al.*, 2011; Liu *et al.*, 2013). Next, a quality control sample comprising of several aliquots of 20 M/mL sperm per aliquot (to perform sperm nuclear protein extraction simultaneously with other samples) to ensure reproducible

and consistent protamine quantification (Mengual *et al.*, 2003; Aoki *et al.*, 2005a; Liu *et al.*, 2013) was not applied in this project due to restriction in the number of mice culled for sperm extraction. Instead, nuclear protein extracted from Batch # 2 mouse was used to generate standard regression lines and aliquots of this sample were applied to each gel as a standard control for validation. Verification of protamine bands separated in acid-urea gel electrophoresis is important to ensure the quantified bands are the expected target proteins which is P1 and P2, and not any other unexpected proteins. Most studies commonly used Western blotting only for verification of P1 and P2 bands and not for quantification (Carrell & Liu, 2001; Hammadeh *et al.*, 2010). However, LC/MS verification, as used in this chapter was not performed in most studies. Therefore, protamine quantification and P1:P2 ratio evaluation using acid-urea gel electrophoresis for clinical application may required both Western blot and LC/MS to ensure specificity.

3.6 Summary and future directions

Mounting evidence highlights the clear link between abnormal protamine ratio in sperm and male infertility, particularly in relation to abnormal semen parameters, increased DNA fragmentation leading to poor fertilisation rate, and abnormal embryonic development (Oliva, 2006; Carrell *et al.*, 2007; Francis *et al.*, 2014). Thus far, most studies have applied a direct assay to quantify protamine levels and P1:P2 ratio using acid-urea gel electrophoresis. Protamines are very small in molecular mass and a highly basic nuclear protein with a unique composition of amino acid residues. Protamines are highly positively charged and separate efficiently in the reducing acidic conditions of an acid-urea gel, based upon molecular charge and mobility (Spiker, 1980; de Yebra & Oliva, 1993; Liu *et al.*, 2013).

While previous studies focused upon protamine quantification in human sperm using acid-urea gel electrophoresis (Balhorn *et al.*, 1988; de Yebra *et al.*, 1998; Mengual *et al.*,

2003; Torregrosa *et al.*, 2006; de Mateo *et al.*, 2009; Garcia-Peiro *et al.*, 2011). To our knowledge, we were the first to apply the same technique to quantify protamine in mouse sperm, predominantly aimed to investigate the effect of cryopreservation upon the total levels of protamine. This represented a novel approach compared to other studies, and the mouse model was selected to test the hypothesis prior to extending such investigations to human sperm. In Chapter 2, we attempted to perform protamine quantification using an immunofluorescent method because it may represent an efficient and robust technique for clinical application which would enable the quantification of protamine in individual sperm and allow visualization and determination of protamine localisation, especially in cryopreserved sperm. Both methods clearly demonstrated the significant effect of cryopreservation upon the total levels of protamine in mouse sperm. As shown in this study and those published earlier, the exact significance of the P1:P2 ratio remains elusive due to the notable variation observed (Nanassy *et al.*, 2011).

While the sufficiency of both P1 and P2 is required for successful fertilisation and embryonic development (Cho *et al.*, 2001; Cho *et al.*, 2003), it is possible that the quantification of P1 and P2 levels separately may represent a better diagnostic marker rather than the P1:P2 ratio. The evaluation of P1:P2 ratio using immunocytochemistry could be possible if co-localisation of P1 and P2 is performed simultaneously in individual sperm, but this is likely to be technically challenging. Immunocytochemistry may be indicated as a better tool for clinical application as acid-urea gel electrophoresis can be time consuming and requires strict verification of proteins bands identity. Moreover, immunocytochemistry enables protamine evaluation in individual sperm cells which structure, DNA integrity, and localisation can be evaluated simultaneously within a shorter period of time. Fundamentally, such applications are not only beneficial in evaluating protamine levels and predicting the genetic integrity of sperm prior to clinical use but also in cryopreserved sperm from SSR

patients or sperm donors. Our results from both Chapter 2 and the present chapter clearly showed the significant effect of cryopreservation upon the total levels of protamine. Worryingly, this significant effect was shown in healthy mouse sperm retrieved via swim-out methodology. Therefore, it is relevant to suggest that such an effect may also occur in healthy human sperm during ART. Since this effect was clearly evident in healthy mouse sperm, this effect is likely to be greater in infertile human sperm, or sperm from SSR patients, who are required to cryopreserved their sperm for latter use. This, however, requires further investigation in a dedicated study.

Chapter 4

The oocyte activation factor, Phospholipase C Zeta (PLCζ): Clinical screening and case studies

4.1. Introduction

The fundamental importance of PLCζ in the activation of oocyte upon gamete fusion was discussed at length in Chapter 1. Evidence clearly shows that abnormal levels or localisation patterning, or genetic mutation, of PLCζ can lead to OAD or infertility. The critical role of PLCζ in the activation of an oocyte has led investigators to focus upon PLCζ as a therapeutic activation agent capable of inducing Ca^{2+} oscillations that closely mimic those seen during normal fertilisation. This would avoid any potential problem associated with the use of existing AOA's. The injection of PLCζ cRNA into mouse and human oocytes already has been demonstrated to be effective in triggering Ca^{2+} oscillations and oocyte activation leading to embryonic development up to the blastocyst stage (Cox *et al.*, 2002; Saunders *et al.*, 2002; Rogers *et al.*, 2004). However, the use of PLCζ cRNA as a clinical oocyte activation agent is not recommended due to uncontrollable translational activity and endogenous reverse transcriptase activity within the oocyte (Spadafora, 2004). Moreover, cRNA is difficult to synthesize in a consistent manner and is very unstable even when stored at -80°C.

Therefore, a purified form of PLCζ recombinant protein is likely to serve as the most appropriate physiological agent to be used to rescue oocyte activation failure and represent a much safer and endogenous alternative to AOAs. Three research groups have now shown successful oocyte activation and subsequent embryonic development in mouse and human oocytes following the injection of purified human PLCζ recombinant protein (Yoon *et al.*, 2012; Nomikos *et al.*, 2013a). While these studies may represent a major breakthrough in the potential clinical application of PLCζ as a physiological oocyte activator (Nomikos *et al.*, 2013a), it is highly prudent to extrapolate such findings into large-scale human studies prior to therapeutic utilisation (Amdani *et al.*, 2013). We still know very little about the molecular biology and physiology of PLCζ in a clinical context. The injection of purified recombinant

human PLCζ was able to rescue oocyte activation failure and induced Ca^{2+} oscillations in mouse oocytes that had previously been injected with mutated forms of PLCζ (Nomikos *et al*., 2013a). Researchers now aim to test PLCζ protein clinically in human oocytes and begin translating this new diagnostic and therapeutic agent to the clinic.

However, there are several features of PLCζ that remain the source of much debate and speculation. These issues must be addressed before clinical translation. For example, the precise pattern of Ca^{2+} oscillations evident upon fertilisation varies between species and may be attributed to the changing levels and/or localisation pattern of PLCζ in sperm from different species, which may determine the degree of PLCζ accessibility/diffusion into the oocyte (Kashir *et al.*, 2014). Moreover, the sensitivity of an oocyte towards the potency of PLCζ to trigger Ca^{2+} oscillations may also vary between species (Kashir *et al.*, 2014). Such variation in the mechanism of action between PLCζ potency and oocyte sensitivity has led to the hypothesis that an unknown oocyte factor is required to activate of PLCζ (Swann & Lai, 2013). This has drawn much interest among researchers and plans are afoot to investigate the involvement of a potential factor, presumably localised within the oocyte, that may associate with PLCζ in order to activate the oocyte (Yu *et al.*, 2012). In fact, this may also explain the inactive state of PLCζ in sperm, which only becomes active in the egg following gamete fusion (Amdani *et al.*, 2013; Nomikos *et al.*, 2013b).

Since the sperm-induced activation of oocytes via Ca^{2+} release occurs following gamete fusion, it is essential that PLCζ is localised in a readily accessible region within the sperm head to allow immediate diffusion of the protein into the oocyte (Kashir *et al.*, 2014). However there is currently much debate among researchers as to the precise localisation of PLCζ in sperm, especially in human sperm. Several immunohistochemistry studies have demonstrated PLCζ localisation in distinct regions of human, mouse and hamster sperm (Grasa *et al.*, 2008; Young *et al.*, 2009).

While different patterns of PLCζ localisation may suggest an involvement in differential functional roles apart from oocyte activation (Fujimoto *et al.*, 2004; Grasa *et al.*, 2008; Kashir *et al.*, 2013b) the topic is still the source of much debate. However, the presence of either single, or multiple populations of PLCζ within a sperm head may determine the robustness of such sperm to trigger the onset and prolonged series of Ca^{2+} oscillations following gamete fusion (Kashir *et al.*, 2014). Consequently there is much continued interest in this area.

The morphological characteristics of a sperm may also be associated with the precise localisation of PLCζ within the sperm head. Several studies have demonstrated deficiency and total abolishment of PLCζ in morphologically abnormal sperm such as globozoospermia (Yoon *et al.*, 2008; Heytens *et al.*, 2009), and pyriform or amophorus-headed sperm (Lee *et al.*, 2014). Such morphological abnormalities could cause distortion of PLCζ within the sperm head, thus compromising oocyte activation ability (Kashir *et al.*, 2012c; Kashir *et al.*, 2014). Therefore, the further assessment and quantification of PLCζ localisation in morphologically abnormal sperm is vital to determine the functional role and capability of such sperm to activate the oocyte.

It is thus very prudent to conduct a large clinical PLCζ screening project in fertile and infertile men across differential bio-geographical populations to improve our understanding of the precise functional role of PLCζ and how abnormalities of PLCζ may impact upon infertility. Our present understanding is not likely to be sufficient enough to deploy a PLCζ protein clinically. Additionally, it is important to expand research to studying the precise Ca^{2+} oscillatory patterns exhibited by sperm from different men and correlates this data with the observed variation in PLCζ levels and localisation patterns. In a previous study, Kashir *et al.* (2013b) clearly demonstrated significant variability in the total levels of PLCζ and proportion

of sperm containing PLCζ in fertile controls and infertile patients (Kashir *et al.*, 2013b). It remains undetermined as to which possible mechanisms underlie such variability.

Mounting research in the development and implementation of PLCζ as a therapeutic tool for OAD and ICSI failure may therefore be able to increase fertilisation rates and assist couples who currently have no alternative but to use AOAs as a potential treatment. Currently, the success rate of ART rarely exceeds 40%. This is very low and can be attributed to various factors, including iatrogenic (procedure-induced) damage (Yelumalai *et al.*, 2012) incurred in the IVF environment and involving gamete manipulation, preservation, or surgical procedures such as vasectomy/vasectomy reversal. Such techniques may exert detrimental effects upon key-sperm proteins, for example protamine, as shown in Chapters 2 and 3 of this thesis. Further supporting evidence has been demonstrated by Kashir *et al.* (2011a), who reported significantly reduced levels of PLCζ in the sperm of fertile men following cryopreservation. These findings do not only suggest the adverse effect of cryopreservation upon this important protein, but also the consequences of using sperm with reduced levels of PLCζ for IVF/ICSI and how this might lead to changes in Ca^{2+} release and thus activation success.

While, the increased risk of infertility with advancing maternal age is well established (Humm and Sakkas, 2013), it is evident that increasing age can also exert a certain degree of infertility risk in males (Belloc *et al.*, 2014) such as changes in the production of reproductive hormones and abnormal semen parameters. Moreover, current research is focusing upon male infertility in relation to increased sperm DNA fragmentation levels (Humm & Sakkas, 2013), which led to the clinical utilisation of DNA fragmentation testing as an additional clinical tool (Nanassy *et al.*, 2011). Intriguingly, studies have revealed a strong correlation between advanced male age and higher DNA fragmentation levels (Das *et al.*, 2013). These findings clearly indicate the potential genetic risk of using sperm from aging men for ART. These

findings also suggest that sperm function may also be comprised with increasing male age, thus affecting reproductive outcome. The underlying mechanism of age factors in relation to male fertility risk remains undefined. However, it is critical to investigate such issues. Whether advancing male age influences the expression and function of key sperm proteins such as protamine or PLCζ, also remains undetermined. This is a significant shortfall as evidence suggests a strong link between the deficiency of these proteins and male infertility (Amdani *et al.*, 2013; Francis *et al.*, 2014). Therefore, it is highly prudent to investigate if men of an older age encounter fertility problems associated with age-related changes of structure expression or function of critical sperm proteins. However, thus far, studies have failed to investigate the potential effect of male age upon PLCζ and whether advancing paternal age may influence oocyte activation ability by affecting PLCζ expression and function.

Since the discovery of PLCζ in 2002 (Saunders *et al.*, 2002), a significant body of research has demonstrated the fundamental role of PLCζ as the sperm-specific oocyte activation factor with strong evidence to support a link between PLCζ deficiency and male infertility (Kashir *et al.*, 2013b). Current research is understandably focussed upon designing therapeutic and diagnostic applications for PLCζ and to translate such technology to the clinics as seen as possible (Amdani *et al.*, 2013; Kashir *et al.*, 2013b; Nomikos *et al.*, 2013a). However, before this happens, there are many outstanding questions which need to be addressed. Firstly, it is vital to enhance our current research PLCζ screening programme to include a much larger number of control and infertile men. This will allow us to evaluate the distribution and localisation patterns of PLCζ in such men, and allow us to investigate, more robustly, the variance in expression levels that were demonstrated in earlier literature. In addition it is important that data are correlated to clinical histories and previous ART outcomes. Such data are also important in that they may link to other projects in the Coward

Laboratory aiming to identify genetic abnormalities in the PLCζ gene. Furthermore, analysis of a larger cohort of patients should allow us, for the first time to investigate whether levels and localisation patterns of PLCζ are related to the age of male patients attending the clinic. This is important for us to ascertain as ART clinics treat a wide age range of male patients but at present do not know whether standard techniques such as IVF or ICSI are the most appropriate treatment for OAD and ICSI failure across all age groups.

In this chapter, I therefore aimed to enhance our clinical knowledge of PLCζ by using an established PLCζ immunofluorescent assay to investigate expression levels and localisation pattern in a large cohort fertile men (controls) and infertile men (patients) from two ART clinics in the UK. During the course of this research, several special cases were identified, which are presented individually in **Section 4.4**.

4.1.1 Specific aims of this chapter

1. Determine the proportion of sperm exhibiting PLCζ and the total levels of PLCζ in sperm from fertile controls (n=13) and infertile patients (n=17).
2. Evaluate and compare the precise localisation patterning of PLCζ in sperm from fertile controls (n=13) and infertile patients (n=17).
3. Design a scoring system based upon the total levels of PLCζ and proportion of sperm containing PLCζ in order to correlate such data with clinical histories and outcomes.
4. Identify and describe specific case studies of interest by demonstrating their clinical parameters, fertilisation history and treatment outcome in relation to PLCζ immunofluorescent analysis and on-going PLCζ genetic screening carried out in Dr Coward's laboratory.
5. To perform comparative analysis between the total levels of PLCζ and proportion of sperm exhibiting PLCζ in different categories of male age in a total of 46 men.

4.2 Materials and Methods

4.2.1 Patient selection and ethics

A total of 30 human semen samples were obtained from fertile controls (n=13) (**Table 4.1**) and infertile patients (n=17) (**Table 4.2**), recruited by the Oxford Fertility Unit (OFU) and Assisted Conception Unit/Medical Research Institute (ACU), Dundee University (United Kingdom). In addition, one patient was recruited by the Department of Reproductive Medicine at Ghent University (Belgium) and presented as a special case study in **Section 4.4.4**. Quantitative PLCζ immunofluorescence data from a total of 16 men (10 controls and 6 patients) previously recruited by the OFU and analysed by a former group member (Dr Junaid Kashir) was additionally included in the current chapter for age analysis. These additional data, in combination with the current study, which provided a collective dataset from a total of 46 men controls (n=23), patients (n=23), are presented only in **Section 4.7**. The remainder of the chapter refers to the 30 new patients recruited during the course of the present study.

Fertile controls were classified as males having no history of infertility or oocyte activation deficiency, or men whose sperm were able to fertilize an oocyte following ART procedures or had fathered a child via natural conception. Selection criteria for infertile men (patients) were determined based upon sperm parameter analysis, such as progressive motility (a+b)% and concentration, (M/ml) (**Table 4.2**), fertilisation rates, history of IVF/ICSI failure or recurrent ICSI failure, men whose sperm were associated with oocyte activation issues as indicated by medical consultants, or sperm morphological abnormalities such as globozoospermia. All samples were provided following a minimum abstinence period of 3 days and seminal parameters were assessed following DGW in the OFU or ACU, according to World Health Organization (WHO, 2010) guidelines. All men provided informed written

consent and the research study was approved by the National Research Ethics Service (South Central Oxford Committee C; Reference number: 10/H0606/65) and Oxford Tropical Research Ethics Committee (OXTREC: 31/09).

4.2.2 Sperm preparation and density gradient washing (DGW)

Semen samples obtained from the OFU were subjected to DGW (**Figure 4.1**), prior to immunofluorescent analysis. Approximately 2ml of 80% PureSperm gradient media was placed into the bottom of a 15mL Falcon tube, and overlaid with 2ml of 40% media (PureSperm 40/80, Nidacon International AB). Subsequently, 1.5ml of liquefied semen was layered on top of the gradient and centrifuged at 300*g* for 20 minutes at room temperature (RT). The supernatant was discarded leaving approximately 0.5ml of pellet at the bottom of tube, which contained the most motile sperm. The pellet was then transferred to a clean Falcon tube containing 5ml of PureSperm Wash (Nidacon International AB) and centrifuged at 500*g* for 10 minutes at RT. The supernatant was discarded and the pellet re-suspended in 200µl of PBS. The sperm suspension in PBS was then washed by centrifugation at 800*g* for 3 minutes and the supernatant discarded without disturbing the pellet. Finally, the sperm pellet was fixed with 100µl 4% PFA for 10 minutes and then stored in 200µl PBS. All sperm samples recruited from the ACU in Dundee were subjected to DGW and PFA fixation onsite in Dundee, and transported to the research laboratory in Oxford for further experimental analysis.

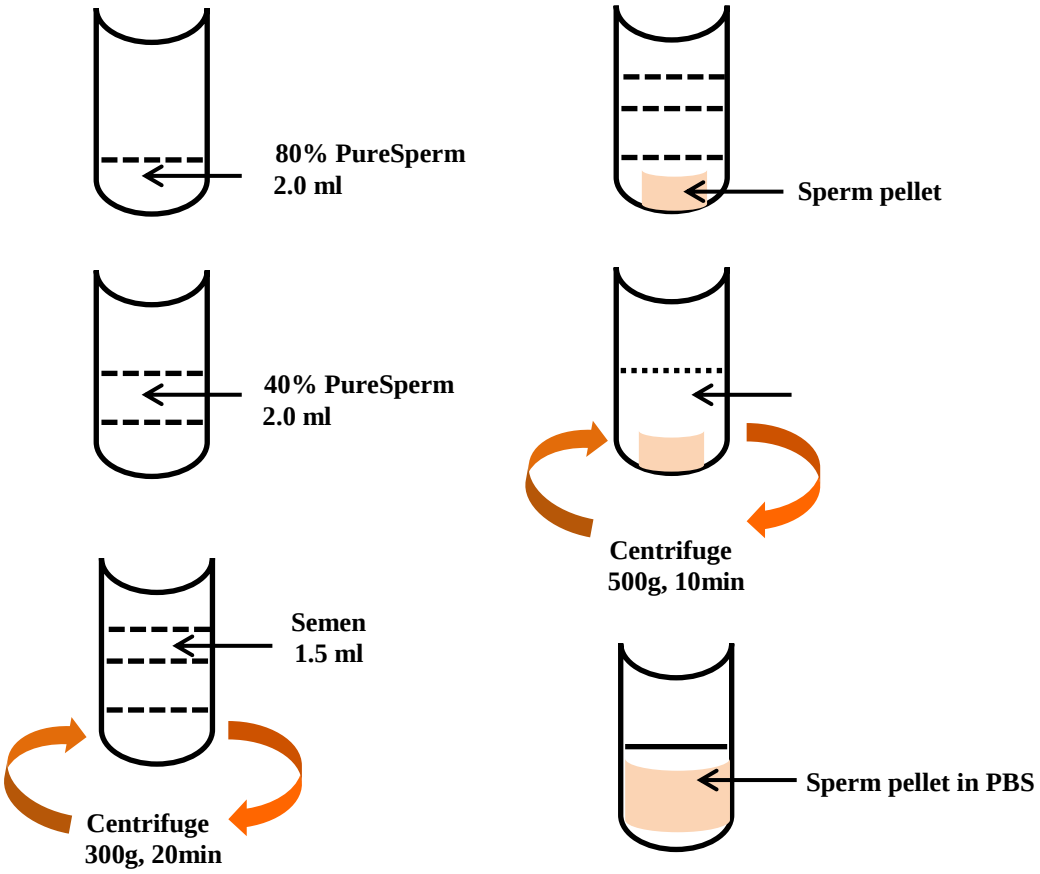


Figure 4.1: Schematic representation of the density gradient washing (DGW) process used for semen preparation to obtain the most motile sperm. The sperm pellet was stored in PBS prior to immunofluorescent analysis.

4.2.3 PLCζ quantitative immunofluorescence assay

4.2.3.1 Anti-PLCζ antibody purification and specificity testing

The human PLCζ antibody used herein was purified using two immunogenic peptides identified in the human PLCζ amino acid sequence (Accession number: AF532185) C-RESKSYFNPSNIKE-coNH_2; C-ETHERKGSDKRGDN). The antibody was produced by injecting the two PLCζ peptides into a rabbit to enable the production of polyclonal antibody (Covo-lab, UK). The antibody was then purified in Oxford from rabbit sera using the immunogenic peptides and a SulfoLink Kit (Pierce Biotechnology, Rockford, USA) as described by Grasa *et al*. (2008). Specificity of the antibody was determined using excessive amounts of peptides to block the reactivity of the purified human PLCζ antibody during

immunocytochemistry, as demonstrated previously by Grasa *et al*. (2008). This antibody has been used in several previous studies arising from the Parrington and Coward Laboratories in Oxford (Grasa *et al.*, 2008; Kashir *et al.*, 2013b) and the de Sutter laboratory in Belgium (Heytens *et al*., 2009).

4.2.3.2 Immunofluorescent staining of PLCζ

Approximately 100µl of sperm suspension was added into PAP-moulds on slides pre-coated with 0.01% poly-L-lysine and permeabilised overnight with 0.5% Triton X-100 in PBS (Sigma Aldrich, UK) as described earlier in Chapter 2 (**Section 2.2.3.1**). Following a 1 hour blocking step with 3% BSA, slides were incubated overnight at 4°C with 100µl of 25µg/ml anti-PLCζ antibody (Cova-Lab, UK) diluted in 0.05% BSA as described by Grasa *et al*. (2008), Heytens *et al*. (2009), and Kashir *et al*. (2013b). Samples were then washed in PBS and incubated at room temperature for 1 hour with 5µg/ml of secondary fluorescent antibody (Alexa Fluor 488 F $[ab']_2$ fragment goat anti-rabbit IgG; Invitrogen, UK). Finally, slides were washed thrice with PBS and mounted with 3-5µl Vectashield H-1200 mounting medium for fluorescence containing DAPI (Vector, UK) and covered with 20mm x 20mm glass cover slips. Slides were left in the dark to await imaging.

4.2.3.3 Microscopic imaging of PLCζ immunofluorescence

PLCζ expression and localisation in sperm was identified using a fluorescence microscope (80*i*, Nikon, UK) and a fluorescein isothicyanate filter (FITC; wavelength 488nm) for green PLCζ fluorescence using an exposure time of 400ms. Slides were imaged at 40x magnification. Additional representative confocal images were also captured at 100x magnification using an Olympus Fluoview FV1000 inverted IX81 confocal laser microscope (Department of Pathology, University of Oxford). Images were acquired at wavelengths of 488nm for green and DIC channels were used for bright field imaging.

4.2.3.4 Sperm selection, quantification and image analysis

The proportion (%) of sperm exhibiting PLCζ, and the mean total levels of PLCζ fluorescence intensity within the sperm [quantified as arbitrary units (AU)] were quantified using Image J software (National Institute of Health, USA) as described in Chapter 2 (**Section 2.2.7**). The localisation pattern of PLCζ was analysed and characterised into several different classifications as described previously by Grasa *et al*. (2008) and Kashir *et al*. (2013b). At least 100 sperm from each sperm suspension from fertile controls (n=13) and infertile patients (n=17) were analysed and quantified for these analyses. Additionally, the standard error of the mean (SEM) was determined for each parameter prior to statistical analysis.

4.2.3.5 Statistical analysis

Data were analysed using GraphPad Prism Software (Version 5, USA) and IBM SPSS 20.0 for Windows (IBM Corp.; Chicago, Illinois, USA). A P-value ≤ 0.05 was considered to be statistically significant. Data were first analysed using the Shapiro-Wilks test to check for a normal distribution across numerical values. Secondly, the homogeneity of variances (homoscedasticity) was assessed by Levene's test. When data did not match parametric assumptions, they were normalized using an Arcsine root transformation (arcsin $\sqrt{x}$).

Classification of controls and patients into statistical clusters

Controls and patients were also classified into clusters. For this purpose, two separate hierarchical cluster analyses were run using the between-groups linkage method based on the squared Euclidean distance. In the first instance, the total levels of PLCζ and proportions of sperm exhibiting PLCζ were used as variables, whereas the second approach used the different localisation patterns as variables. Total levels of PLCζ, proportions of sperm

exhibiting PLCζ, and different localisations were also evaluated using One-Way Analysis of Variance (ANOVA) followed by the Bonferroni post-hoc test for pairwise comparisons.

Comparison between all controls and patients

Differences between all controls and patients in total levels of PLCζ, proportions of sperm exhibiting PLCζ, and different localisation patterns, were evaluated with an unpaired Student's *t*-test.

Comparisons in case studies

Proportions of spermatozoa exhibiting PLCζ were compared in specific case studies (**Section 4.4**) using Fisher's Exact test. Total levels of PLCζ were compared with an unpaired Student's *t*-test.

Evaluation of the age on PLCζ levels and proportions of sperm exhibiting PLCζ, and localisation patterns

Finally, the effects of age group upon the total levels of PLCζ, proportions of sperm exhibiting PLCζ, and different localisations were evaluated using One-Way Analysis of Variance (ANOVA) followed by the Bonferroni post-hoc test for pairwise comparisons.

4.2.4 Genetic analysis and screening of the PLCζ gene promoter region

Data related to the genetic screening of PLCζ and single nucleotide polymorphism (SNP) detection described herein **Chapter 4,** and **Appendices 1.0** and **2.0**, was generated and provided for inclusion in this thesis, with consent, by Miss Siti Nornadhirah Amdani (DPhil Student, Nuffield Department of Obstetrics and Gynaecology, University of Oxford). This information is specifically described in **Sections 4.2.4**, **4.4.2**, **4.4.4** and **Figures 4.18** and **4.25**. Methodology used for SNP detection and analysis is described in **Appendices 1.0** and **2.0**.

In brief, the promoter-exon 1 region of the PLCζ gene was amplified from genomic DNA extracted from the saliva of selected patients of interest (patient 7 and patient PM). Following PCR amplification, the DNA amplicon was sent for sequencing (Geneservice, Oxford). Abnormalities in the resulting sequences were identified by comparing with the wild type PLCζ sequence obtained from Mutation Discovery Software (www.mutationdiscovery.com) and sequence alignment was performed by ClustalW Multiple Sequence Alignment application (Bioedit Sequence Alignment Editor Software).

4.3 Results

4.3.1 Patient characteristics and sperm analysis

The total number of samples received from the OFU (n=18) and ACU (n=12) were divided into two groups: fertile controls (n=13; **Table 4.1**) and infertile patients (n=17; **Table 4.2**). Age of the controls and patients ranged from 32 to 48 years, and from 31 to 49 years, respectively. Routine clinical sperm parameters (eg. sperm motility and concentration) was assessed in all 13 controls and 17 patients following density gradient washing (DGW) at the OFU and ACU clinics. Motile sperm count in the 13 controls ranged from $5.6x10^6$ sperm/mL to $41.0x10^6$ mL, with a mean of $16.7x10^6$ sperm/mL. Progressive sperm motility (a + b) %, among the controls ranged from 45% to 95%, with a mean of 77.5% (**Table 4.1**).

Table 4.1: Summary of male age, sperm morphology, motility grades, and motility count following density gradient washing (DGW), and outcome of assisted reproductive technology (ART) procedure used in thirteen controls.

			Motility after DGW%		Treatment	Oocytes	Diagnosis
Controls (n=13)	**Sample source**	**Male age**	**Progressive motility a + b (%)**	**Motile count (10^6/mL)**	**IVF/ICSI**	**Number of oocytes fertilised (%)**	
1	OFU	38	81	13.8	IVF	6/7 (86)	Unexplained
2	OFU	41	72	10.6	IVF	2/2 (100)	POR
3	OFU	39	94	6.3	IVF	6/12 (50)	PCOS
4	OFU	42	81	32.4	IVF	1/1 (100)	Endometriosis
5	OFU	32	95	16.2	IVF	11/14 (79)	Unexplained
6	OFU	39	62	16.0	IVF	1/1 (100)	Unexplained
7	OFU	34	79	19.8	IVF	6/6 (100)	Unexplained
8	OFU	34	81	20.3	IVF	2/6 (33)	POR
9	OFU	40	86	12.9	IVF	4/7 (57)	Unexplained
10	OFU	37	87	15.5	IVF	2/5 (40)	N/A
11	OFU	35	91	41.0	IVF	2/2 (100)	N/A
12	OFU	48	54	6.1	ICSI	6/8 (75)	N/A
13	OFU	43	45	5.6	ICSI	4/5 (80)	Unexplained

POR = Poor ovarian reserve
PCOS = Polycystic ovarian syndrome
N/A = Not available

In the patient group, total motile sperm count ranged from $1.7x10^6$ mL to $44.4x10^6$ sperm/mL with a mean of $16.0x10^6$ sperm/mL. Progressive sperm motility (a + b) %, ranged from 9% to 91% with a mean of 61.4% (**Table 4.2**). Total motile sperm count in the patient group was significantly lower ($P \leq 0.05$; as indicated by the Student's *t*-test) than in the controls, even though progressive motility was comparable between the two groups. Sperm from all men (controls and patients) exhibited normal morphology, except for patient 6. This particular patient had experienced surgery for vasectomy reversal and was attending ACU for infertility treatment. Sperm from patient 6 exhibited morphology abnormalities in the head region and is described in detail in **Section 4.4.1** of this chapter.

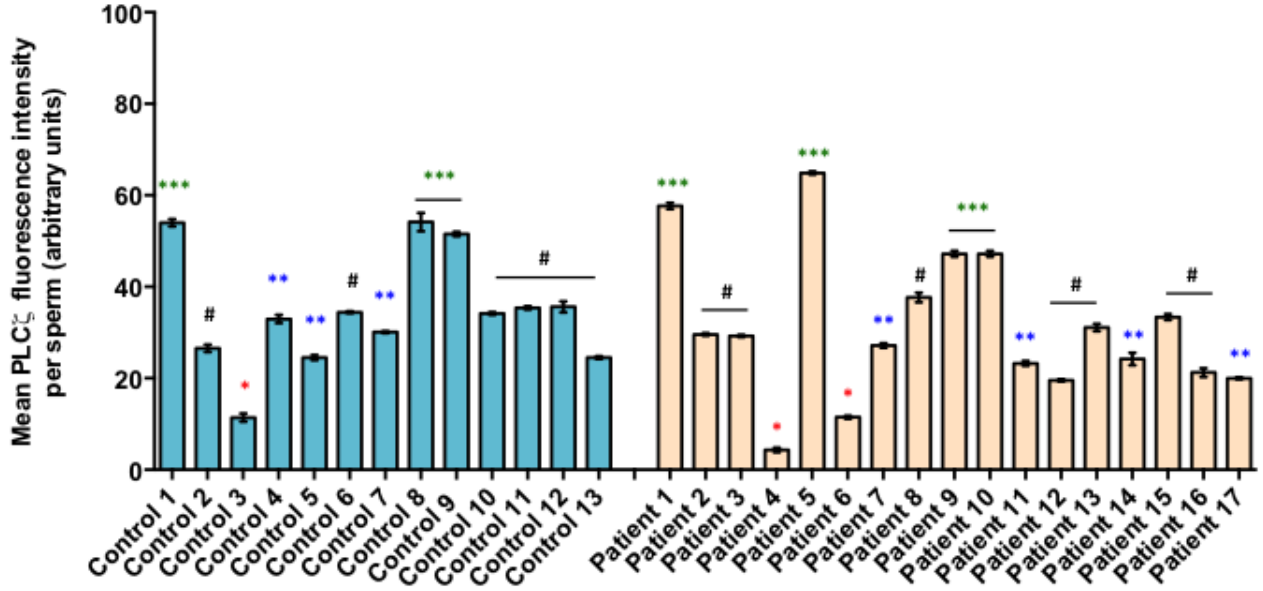
Table 4.2: Summary of male age, sperm morphology, motility grades, and motility count following density gradient washing (DGW), and outcome of assisted reproductive technology (ART) procedure used in seventeen patients.

			Motility after DGW%		Treatment	Oocytes	Diagnosis
Patients (n=17)	**Sample source**	**Male age**	**Progressive motility a + b (%)**	**Motile count (10^6/mL)**	**IVF/ICSI**	**Number of oocytes fertilised (%)**	
1	ACU	35	41	25.0	ICSI	0/8 (0)	Unexplained
2	OFU	41	91	7.6	ICSI	13/13 (100)	Unexplained
3	OFU	40	53	4.2	ICSI	1/7 (14)	Ovarian failure
4	OFU	49	61	1.7	ICSI	0/4 (0)	N/A
5	OFU	36	69	10.4	ICSI	8/9 (89)	Unexplained
6	ACU	39	67	22.3	ICSI	0/10 (0)	Vasectomy
7	ACU	35	38	3.1	ICSI	0/9 (0)	Unexplained
8	ACU	42	86	15.0	ICSI	0/7 (0)	Unexplained
9	ACU	36	72	5.5	IVF	1/11 (9)	Unexplained
10	OFU	45	48	44.4	ICSI	2/15(13)	N/A
11	ACU	34	9	29.4	IVF	0/12 (0)	Unexplained
12	ACU	31	60	19.1	ICSI	1/7(14)	PCOS
13	ACU	35	76	13.9	IVF	2/20 (10)	Unexplained
14	ACU	36	47	3.3	ICSI	0/4 (0)	Unexplained
15	ACU	37	85	27.0	IVF	0/6 (0)	Unexplained
16	ACU	33	71	6.7	IVF	0/6 (0)	Unexplained
17	ACU	41	69	33.4	ICSI	0/12 (0)	PCOS

PCOS = Polycystic ovarian syndrome
N/A = Not available

4.3.2 Comparison of total PLCζ immunofluorescence levels between thirteen fertile controls and seventeen infertile patients

Total mean fluorescence levels of PLCζ in sperm showed variability ($P \leq 0.05$; as indicated by One-Way ANOVA) between each individual control (n=13) and patient (n=17) (**Figure 4.2, Anova table 4.1**). In the controls, the total level of PLCζ ranged from 11.4AU to 54.2AU with controls 3 and 8 exhibiting the lowest and highest levels respectively. The total levels of PLCζ observed in sperm from control 3 were significantly lower ($P \leq 0.05$; as indicated by One-Way ANOVA) as compared to the remaining controls.



Anova table 4.1					
	Sum of Squares	df	Mean Square	F	Sig.
Intersections	416001.6	1	791.4	525.6	0.000
Controls/Patients	58140.8	1	806.7	72.1	0.000
BF_Controls/Patients	169283.9	112	252.6	5.9	0.000

Figure 4.2: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in sperm from 13 controls and 17 infertile patients. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Coloured asterisks (***, **, *) and hash mark (#) denote controls and patients categorised into Cluster 1, Cluster 2, Cluster 4 and Cluster 3 respectively. Statistical analysis showed significant variability ($P \leq 0.05$) between all controls and patients. Data are shown as mean ± SEM.

Among the patients (n=17) (**Figure 4.2, Anova table 4.1**), the total levels of PLCζ varied significantly ($P \leq 0.05$; as indicated by One-Way ANOVA) and fell between a range of 4.3AU to 64.9AU, with patients 4 and 6 exhibiting the lowest, and patient 5 exhibiting the highest, mean fluorescence levels of PLCζ. The total levels of PLCζ in sperm from patient 4 (4.3 ± 0.5AU) and patient 6 (11.5 ± 0.4AU) were statistically significant ($P \leq 0.05$; as indicated by One-Way ANOVA) as compared to other patients.

Further analysis allowed the arbitrary classification of controls and patients into sub-groups (or "clusters") based upon PLCζ immunofluorescence levels and the proportion of sperm exhibiting PLCζ. In order to identify these clusters, the total levels of PLCζ and the proportional data from both control and patient groups were analysed using cluster statistical

analysis. A total of four clusters were obtained (**Table 4.3**). The characteristics of each cluster, in terms of PLCζ levels and the proportions of sperm containing PLCζ, is presented at the end of this section (**Table 4.6**). Proportional PLCζ analysis outcomes and cluster results are described in **Section 4.3.3**.

Table 4.3: Number and proportion of controls and patients classified into each cluster.

Cluster	**1**	**2**	**3**	**4**
Controls	1, 8, 9	4, 5, 7	2, 6, 10 11, 12, 13	3
(n=13)	(n=3)	(n=3)	(n=6)	(n=1)
Percentage (%)	23.0	23.0	46.0	8.0
Patients	1, 5, 9, 10	7, 11, 14, 17	2, 3, 8 12, 13, 15, 16	4, 6
(n=17)	(n=4)	(n=4)	(n=7)	(n=2)
Percentage (%)	24.0	24.0	41.0	11.0
Total (n=30)	**(n=7)**	**(n=7)**	**(n=13)**	**(n=3)**
Percentage (%)	**23.0**	**23.0**	**43.0**	**10.0**

n = Number of controls or patients.

The total levels of PLCζ from controls in Cluster 1 were similar and ranged from 51.2AU to 54.2AU with a mean of 53.2 ± 0.9AU. PLCζ levels did not differ statistically among the controls in this cluster. Similarly, controls in Cluster 2 did not differ significantly and PLCζ levels ranged from 24.9AU to 33.0AU with a mean of 29.3 ± 2.4AU. Controls in Cluster 3 showed levels of PLCζ ranging between 24.5.0AU to 35.6AU with a mean of 31.8 ± 2.0AU. Control 3 exhibited the lowest levels of PLCζ 11.4AU and was categorised into Cluster 4.

Patients in Cluster 1 showed total levels of PLCζ ranging from 47.2AU to 64.9AU with a mean of 56.0 ± 3.7AU. Similarly, patients in Cluster 2 also exhibited similar range with total levels of PLCζ ranging from 20.0AU to 27.1AU with a mean of 23.7 ± 1.5AU. The total levels of PLCζ in patients from Cluster 3 ranged from 19.6AU to 37.7AU with a mean of 28.8 ± 2.4AU. Patients 4 and 6 in Cluster 4 showed the lowest levels of PLCζ with a mean of 9.1 ± 2.9AU.

Comparative analysis revealed that the total level of PLCζ was not significantly different in clusters between controls and patients (**Table 4.4**). For example, total levels of PLCζ from Cluster 1-Control and Cluster 1-Patient were comparable. A similar trend was observed between Cluster 2-Control and Patient, Cluster 3-Control and Patient, and Cluster 4-Control and Patient ($P \geq 0.05$; as indicated by the Student's *t*-test). However, showed statistically significant differences in the total levels of PLCζ between the clusters, indicating that PLCζ may fall within several defined ranges as shown by the four clusters ($P \leq 0.05$; as indicated by One-Way ANOVA).

In summary, these data indicate that the total levels of PLCζ in sperm from certain groups of controls and patients were similar, and that infertile patients do not necessarily exhibit low levels of PLCζ. Collectively, this analysis demonstrated significant variability in total levels of PLCζ samples processed during this study. However, the levels of PLCζ from certain clusters of controls and patients were similar, and fell between specific ranges according to statistical analysis, thus allowing the classification of controls and patients into broad sub-groupings.

Table 4.4: Mean total levels of phospholipase C zeta (PLCζ) exhibited in controls and patients from categorised clusters.

Cluster	1	2	3	4
Controls	53.2 ± 0.9 *, **	29.3 ± 2.4 *	31.8 ± 2.0 **	11.4
Patients	56.0 ± 3.7 *, **, ***	23.7 ± 1.5 *, ***	28.8 ± 2.4 **, ***	9.1 ± 2.9 ***

Asterisks (*, **) and (*, **, ***) denote statistically significant differences ($P \leq 0.05$) between clusters in controls and patients respectively. Total levels of PLCζ were quantified in arbitrary units (AU). Data are shown as mean ± SEM.

4.3.3 Comparison of the proportion of sperm exhibiting PLCζ between thirteen fertile controls and seventeen infertile patients

The proportion of sperm containing PLCζ from each individual control showed significant differences ($P \leq 0.05$; as indicated by One-Way ANOVA) varying from 13% to 98% (**Figure 4.3**). Control 3 exhibited the lowest proportion ($P \leq 0.05$; as indicated by One-Way ANOVA) of sperm containing PLCζ as compared to the remaining controls. Control 3 also exhibited the lowest level of PLCζ (**Figure 4.2**). The proportion of sperm exhibiting PLCζ was highest (98%) in controls 9 and 13. Among the patients, patients 4 and 6 exhibited the lowest (1% and 3%), and patient 8 the highest (99%) number of sperm containing PLCζ. As described in **Section 4.3.2** (**Figure 4.2**), patients 4 and 6 also showed the lowest immunofluorescence levels of PLCζ. The proportion of sperm from patients 4 and 6 exhibiting PLCζ was significantly lower than all other patients ($P \leq 0.05$; as indicated by One-Way ANOVA).

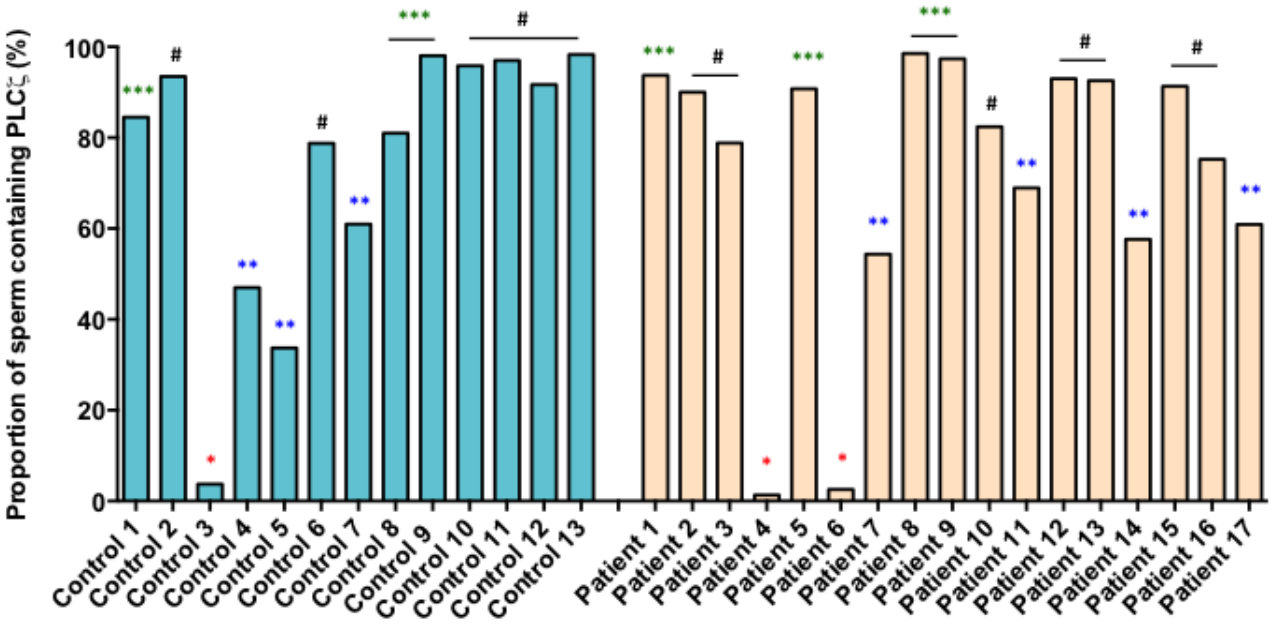


Figure 4.3: Proportion of sperm from 13 controls and 17 infertile patients exhibiting phospholipase C zeta (PLCζ). Number of sperm with PLCζ was determined with the use of ImageJ software. Coloured asterisks (***, **, *) and hash mark (#) denote controls and patients categorised into Cluster 1, Cluster 2, Cluster 4 and Cluster 3 respectively. Statistical analysis showed significant variability ($P \leq 0.05$) between controls and patients. Data are shown as percentages.

The clusters obtained and described based upon total immunofluorescence levels for the controls in **Section 4.3.2** were also applicable for proportional analysis. For example, Cluster 1-Control showed that the proportion of sperm exhibiting PLCζ ranged from 78% to 98% with a mean of 88% and there were no significant differences between the controls in Cluster 1 (**Figure 4.3**). Similarly, the proportion of sperm exhibiting PLCζ from Cluster 2-Control ranged from 40% to 61% with a mean of 44% (**Figure 4.3**). The proportion of sperm exhibiting PLCζ in Cluster 3-Control ranged from 79% to 98% with a mean of 91%. Finally, control 4 was classified into Cluster 4 with only 13% of sperm exhibiting PLCζ. Among the patients, Cluster 1-Patient showed that the proportion of sperm exhibiting PLCζ ranged from 88% to 97% with a mean of 92%. The proportion of sperm exhibiting PLCζ in patients from Cluster 2 was ranged from 46% to 67% with a mean of 57%. Similarly, the proportion of sperm containing PLCζ in Cluster 3-Patient ranged from 75% to 99% with a mean of 89%.

Cluster 4 consisting of just 2 patients, showed the lowest mean proportion of sperm exhibiting PLCζ (2%).

As seen with the comparative analysis of total levels of PLCζ between clusters in controls and patients (**Table 4.4**), the proportion of sperm exhibiting PLCζ was comparable within the same clusters (**Table 4.5**). For example, the proportion of sperm exhibiting PLCζ from Cluster 1-Control and Cluster 1-Patient was comparable. A similar trend was observed between [Cluster 2-Control and Patient], [Cluster 3-Control and Patient] and [Cluster 4-Control and Patient] ($P \geq 0.05$; as indicated by the Student's *t*-test). Finally all data for total levels of PLCζ and proportion of sperm exhibiting PLCζ from all controls (n=13) and patients (n=17) were combined, and subjected to collective cluster analysis (**Table 4.6**).

Table 4.5: Proportion of sperm exhibiting phospholipase C zeta (PLCζ) in controls and patients from categorised clusters.

Cluster	1	2	3	4
Controls	88.0 *	44.0 *, **	91.0 **	13.0
Patients	92.0 *, ***	57.0 *, **, ***	89.0 **	2.0 *, **, ***

Asterisks (*, **) and (*, **, ***) denote statistically significant differences ($P \leq 0.05$) between clusters in the controls and patients respectively. Data are shown as percentages.

Table 4.6: Collective cluster analysis for total levels and proportion of sperm exhibiting phospholipase C zeta (PLCζ) in all controls and patients.

Cluster	1	2	3	4
PLCζ levels	54.8 *, **, ***	26.1 *	30.2 **	16.5 ***
Proportion of sperm with PLCζ	90.0 *, **	51.0 *, ***	90.0 ***	6.0 **, ***

Asterisks (*, **, ***) denote statistically significant differences ($P \leq 0.05$) between clusters. Total levels of PLCζ were quantified in arbitrary units (AU). Proportion of sperm exhibiting PLCζ are shown as percentages.

The mean total levels of PLCζ for each cluster provided the foundation for the design of a novel scoring system (data presented in **Section 4.5**), which could be used to predict the fertilisation outcome of men in a clinical setting.

In this study, the total number of sperm analysed from controls (n=13) and patients (n=17) were (n=1243) and (n=1972) respectively. Mean levels of PLCζ from patients (30.4 ± 4.0AU) were reduced by 12% as compared to controls (34.6 ± 3.5AU) (**Figure 4.4**). Similarly, the mean proportion of sperm exhibiting PLCζ in patients (68%) showed a reduction of 6% as compared to controls (74%) (**Figure 4.5**). However, statistical analysis showed that this reduction in the patient group was not statistically significant ($P \geq 0.05$; as indicated by the Student's *t*-test).

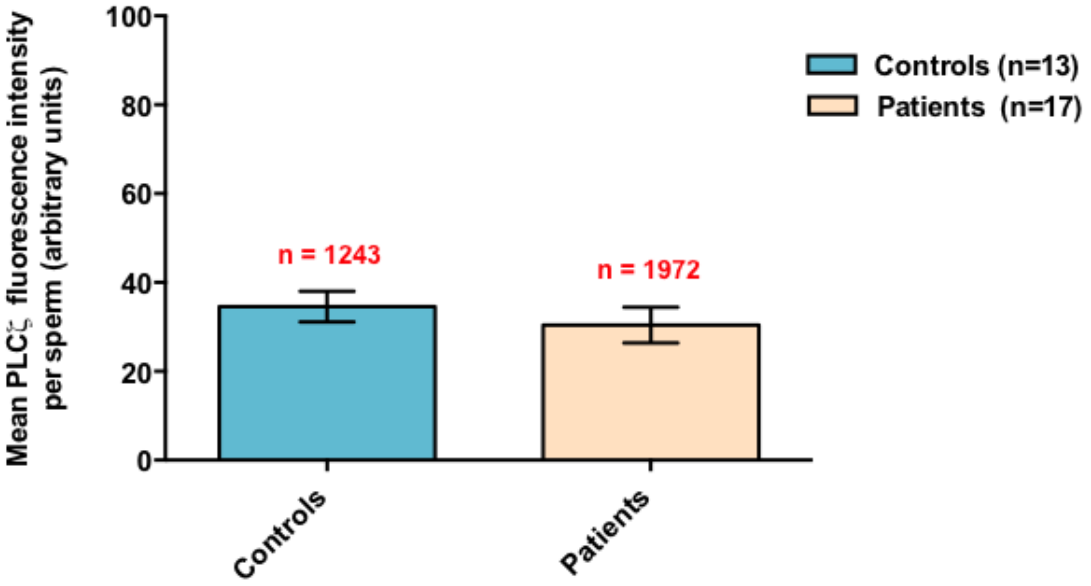


Figure 4.4: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels from 13 controls and 17 infertile patients. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. There were no statistical significant differences in the total levels of PLCζ between controls and patients. The numerical values denoted in red font on each histogram represents the total number of sperm analysed. Data are shown as mean ± SEM.

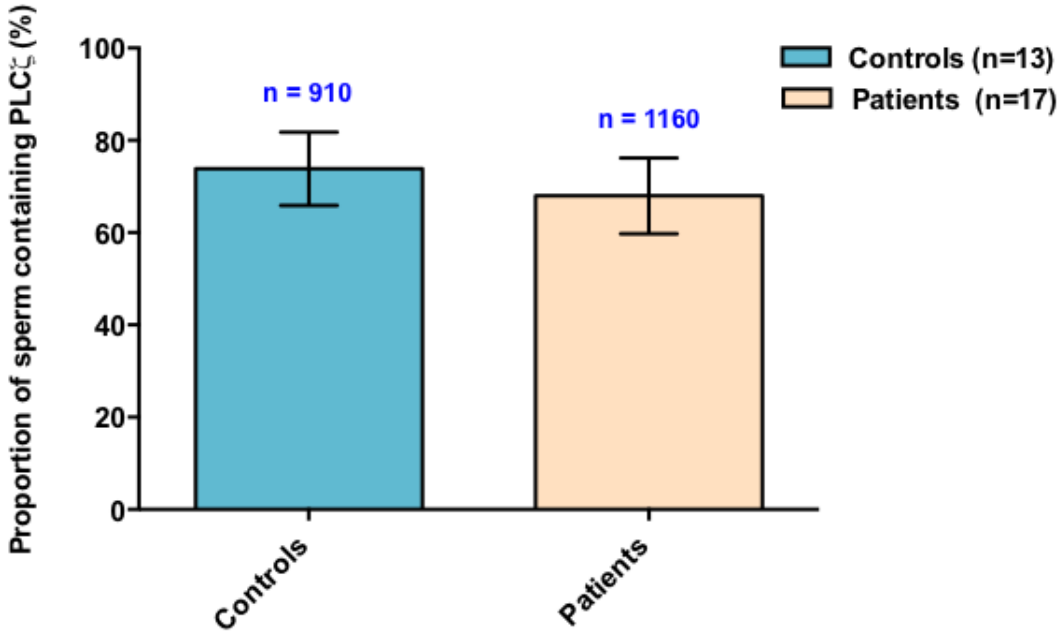


Figure 4.5: Mean proportion of sperm from 13 controls and 17 infertile patients exhibiting phospholipase C zeta (PLCζ). The number of sperm with PLCζ was determined with the use of ImageJ software. There were no statistical significant differences in the number of sperm exhibiting PLCζ between controls and patients The numerical values denoted in blue font on each histogram represents the number containing PLCζ out of the total number of sperm analysed. Data are shown as mean ± SEM.

4.3.4 Localisation patterning of PLCζ in sperm from all thirteen fertile controls and seventeen infertile patients

Here, immunofluorescence analysis was used to compare the localisation of PLCζ in control and infertile sperm. Analysis showed a high degree of variation as previously demonstrated by Grasa *et al*., (2008) and Kashir *et al*., (2013b). Similar to these previous reports, PLCζ was localised to three distinct regions within the sperm head: the acrosomal region (A), the post-acrosomal region (P), or the equatorial (E) region (Grasa *et al*., 2008) (**Figure 4.6**). Further analysis showed that these localisation patterns were present in various combinations such as acrosomal + post-acrosomal (A+P), acrosomal + equatorial (A+E), post-acrosomal + equatorial (P+E), and a combination of all three localisation patterns (A+E+P). Some sperm were totally devoid of PLCζ (denoted hereafter as 'None').

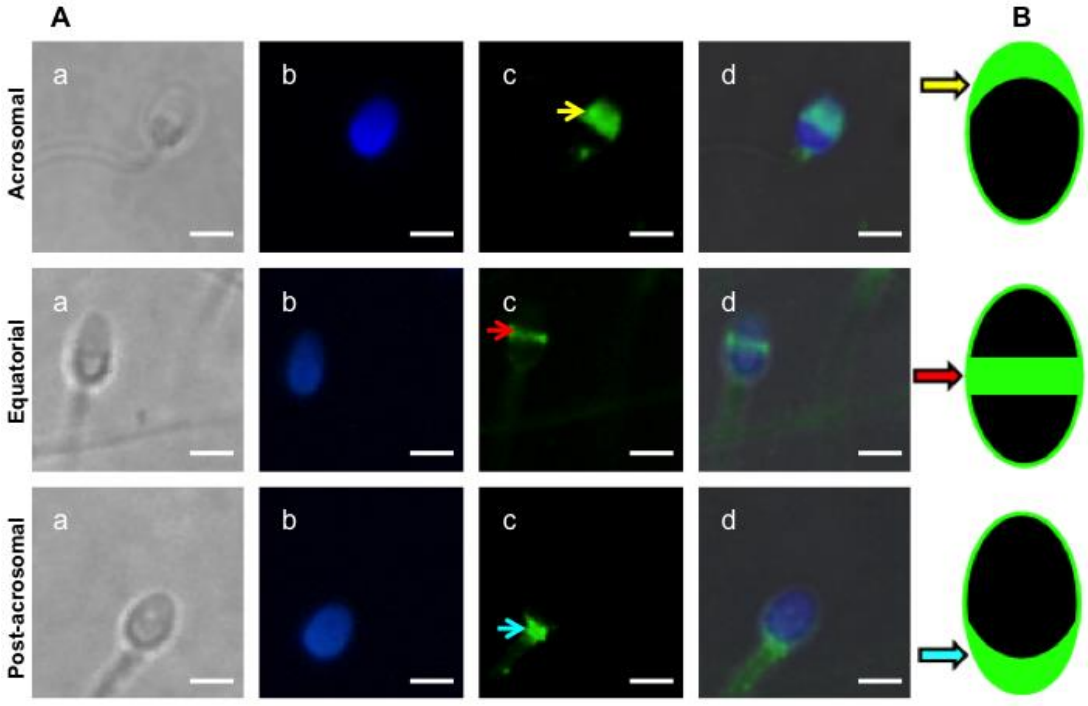


Figure 4.6: (**A**) Microscopic images of (a) bright field, (b) nuclei, (c) phospholipase C zeta (PLCζ), (d) overlay of sperm from a fertile control man, (**B**) schematic images showing three distinct localisation patterning of PLCζ within human sperm head. Yellow arrow indicates acrosomal, red arrow: equatorial and blue arrow: post-acrosomal localisation of PLCζ. White scale bar indicates 5.0µm.

The localisation patterns of PLCζ varied significantly between controls and patients, with P+E and A+E+P revealed as the predominant localisation patterns (**Figure 4.7**). In the controls, 30% and 18% of the total sperm analysed exhibited PLCζ in P+E and A+E+P regions respectively. The same PLCζ patterns were exhibited in 24% and 17% of sperm from the patient group (**Table 4.7**). The P and E populations of PLCζ were also observed frequently in sperm from both the control and patient group but without significant difference observed in 5% to 15% of sperm analysed. The proportion of sperm with PLCζ localised in the P+E region in sperm from patients was significantly lower by 20% ($P \leq 0.05$; as indicated by One-Way ANOVA) compared to controls (**Figure 4.8**). However, the proportion of sperm exhibiting PLCζ in A+E+P regions in controls and patients differed only by 6%. Most notably, however, the proportion of sperm exhibiting complete absence of PLCζ (None) in the patient group was significantly higher by 14% than the control group (**Table 4.7**).

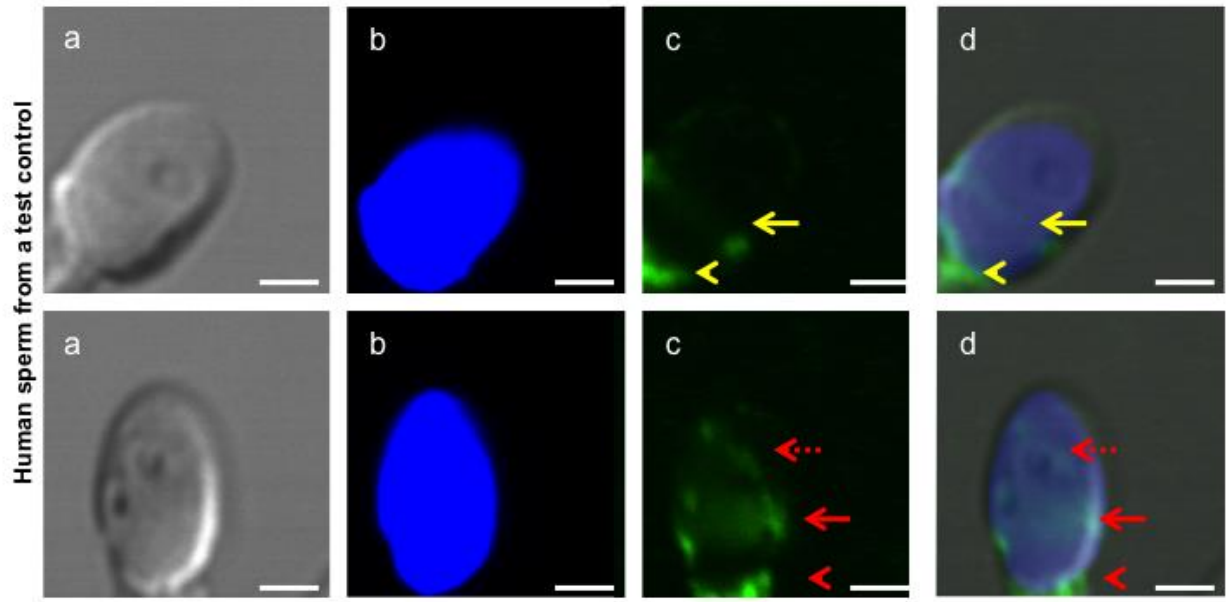


Figure 4.7: Representative confocal images of (a) differential interference contrast (DIC), (b) nuclei, (c) phospholipase C zeta (PLCζ) and (d) overlay showing predominant localisation patterning of PLCζ in human sperm from a test fertile male. Yellow arrow and arrow head indicates PLCζ in equatorial and post-acrosomal region of the sperm head respectively. Red dotted arrow, arrow and arrow head indicates all three patterns (A+E+P) localisation of PLCζ within a single sperm head. White scale bar indicates 2.0µm.

Table 4.7: Number of sperm exhibiting phospholipase C zeta (PLCζ) localisation patterns in controls (n=13) and patients (n=17).

	A (Acrosomal)	P (Post-acrosomal)	E (Equatorial)	A+P	A+E	P+E	A+E+P	None
Controls	15	94	185	9	17	372	218	333
Patients	20	107	182	42	4	464	341	812

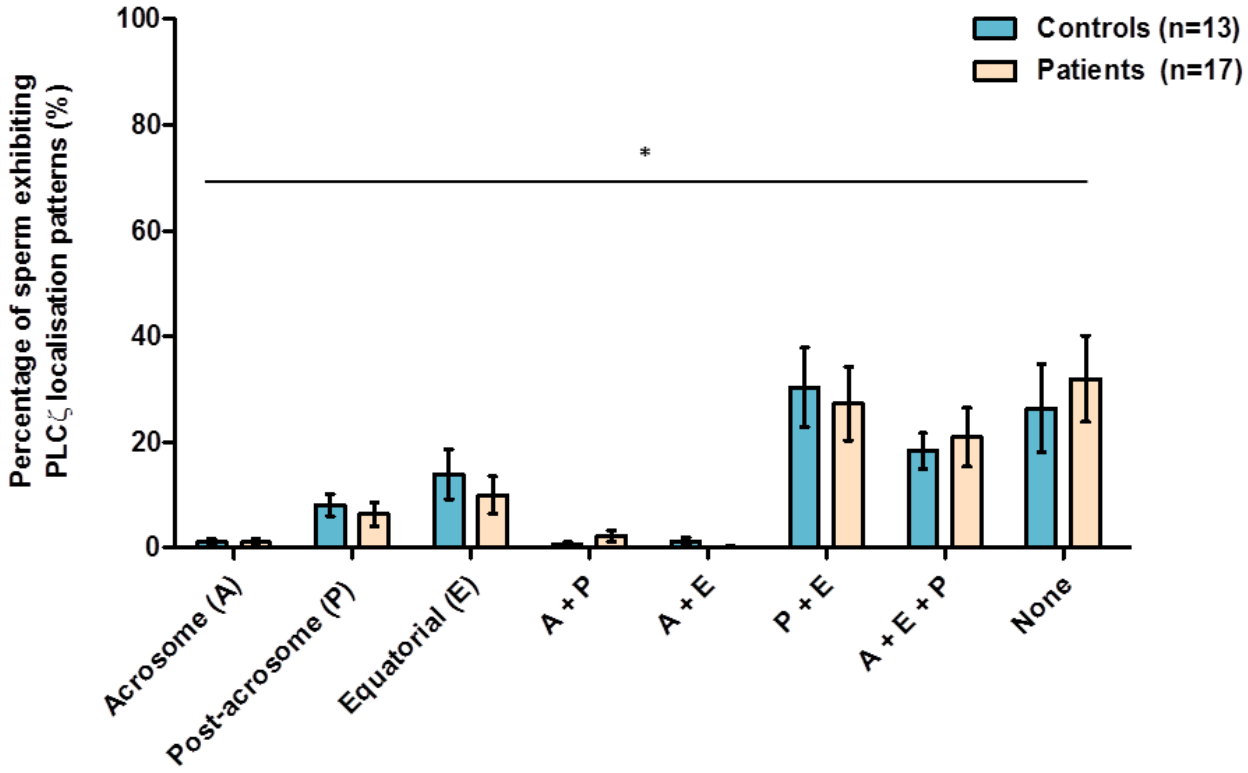


Figure 4.8: Total proportion of sperm from 13 controls and 17 infertile patients containing phospholipase C zeta (PLCζ) in different localisation patterns. Analysis was performed by ImageJ software. Asterisk (*) denotes statistically significant variability ($P \leq 0.05$) for the overall data. Data are shown as mean ± SEM.

4.3.5 Localisation patterning of PLCζ in sperm from each individual control and infertile patient

Here, the localisation patterning of PLCζ in sperm from all controls and patients were classified into groups following cluster analysis (the same cluster analysis performed in **Section 4.3.2**) (**Table 4.8**) to identify the predominant localisation pattern of PLCζ in each individual control (**Table 4.9**) and patient (**Table 4.10**). For example, controls or patients who were classified into Cluster 1 exhibited an equatorial PLCζ pattern as the predominant localisation within the sperm (**Table 4.8**).

Table 4.8: Cluster analysis of the proportion of sperm exhibiting phospholipase C zeta (PLCζ) localisation patterning in all controls and patients categorized into specific clusters.

PLCζ localisation patterns	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Acrosomal (A)	2.0	2.0	0.0	1.0
Post-acrosomal (P)	10.0	11.0	5.0	1.0
Equatorial (E)	**31.0**	4.0	11.0	1.0
A+P	2.0	3.0	2.0	0.0
A+E	2.0	1.0	0.0	0.0
P+E	14.0	11.0	**64.0**	3.0
A+E+P	11.0	**52.0**	7.0	**8.0**
None	**28.0**	16.0	11.0	**86.0**

Numerical value in bold red font indicates the predominant pattern of PLCζ in sperm from controls or patients from their respective clusters. Bold blue font indicates the proportion of sperm that are devoid of PLCζ classified into Cluster 1 and Cluster 4. Data are shown as percentages.

In the control group (**Table 4.9**), the localisation patterns of PLCζ showed variability between each individual. Sperm from most men exhibited different populations and combinations of PLCζ localisations although a single population of PLCζ was identified as the predominant localisation pattern. The P+E population was observed most frequently (46%) in controls according to Cluster 3.

Cluster analysis also revealed that the most common pattern of PLCζ across all controls and patients were E (Cluster 1), A+E+P (Cluster 2) and (Cluster 3) P+E and None (Cluster 4). Controls and patients classified into Cluster 4 showed a large proportion of sperm exhibiting complete absence of PLCζ. For example, 4 controls were classified into Cluster 4 indicating that although there were some sperm showing a predominant localisation of PLCζ, but that a larger proportion of sperm did not contain PLCζ.

Table 4.9: Percentage of sperm exhibiting phospholipase C zeta (PLCζ) localisation patterns in each individual control.

Controls (n=13)	A (Acrosomal)	P (Post-acrosomal)	E (Equatorial)	A+P	A+E	P+E	A+E+P	None
1	7.1	2.7	**36.6**	4.4	7.0	14.2	15.9	12.4
2	0.0	4.0	22.0	0.0	1.0	**47.0**	19.0	7.0
3	1.0	0.0	0.0	0.0	0.0	0.0	**2.0**	**97.0**
4	2.0	7.0	**13.0**	0.0	1.0	7.0	10.0	**60.0**
5	1.0	3.0	0.0	0.0	0.0	8.0	**21.0**	**67.0**
6	3.0	3.0	**49.0**	3.0	2.0	4.0	15.0	21.0
7	0.0	6.0	2.0	0.0	0.0	8.0	**50.0**	**34.0**
8	0.0	27.0	16.7	0.0	5.2	11.5	17.7	22.0
9	0.0	8.0	0.0	0.0	0.0	**72.0**	18.0	2.0
10	0.0	17.0	2.0	0.0	0.0	**60.0**	17.0	4.0
11	0.0	9.0	4.0	1.0	0.0	**67.0**	16.0	3.0
12	0.0	14.7	0.0	0.0	0.0	**41.2**	32.3	11.8
13	0.0	3.0	36.0	0.0	0.0	**55.0**	4.0	2.0

Numerical value in bold red font indicates the proportion of sperm exhibiting a predominant pattern of PLCζ in sperm from each individual control classified according to their clusters. Bold blue font indicates the proportion of sperm devoid PLCζ. Data are shown as percentages.

Table 4.10: Percentage of sperm exhibiting phospholipase C zeta (PLCζ) localisation patterns in each individual patient.

Patients (n=17)	A (Acrosomal)	P (Post-acrosomal)	E (Equatorial)	A+P	A+E	P+E	A+E+P	None
1	12.8	2.8	0.0	13.8	0.9	0.0	**63.3**	6.4
2	0.0	4.0	6.0	6.0	2.0	8.0	**66.0**	8.0
3	0.0	5.0	0.0	13.0	1.0	**54.0**	6.0	21.0
4	0.0	0.0	0.0	1.0	0.0	0.0	0.0	**99.0**
5	2.0	31.0	0.0	2.0	0.0	0.0	**55.0**	10.0
6	0.0	0.0	3.0	0.0	0.0	0.0	0.0	**97.0**
7	0.0	0.0	**44.0**	0.0	0.0	2.0	0.0	**54.0**
8	0.0	0.0	0.0	0.0	0.0	**81.0**	18.0	1.0
9	0.0	16.0	8.0	0.0	0.0	22.0	**42.0**	12.0
10	0.0	26.0	6.0	5.0	0.0	21.0	10.0	**32.0**
11	0.0	3.0	20.0	0.0	0.0	**69.0**	0.0	8.0
12	0.0	12.0	13.0	0.0	0.0	29.0	**36.0**	10.0
13	0.0	0.0	1.0	0.0	0.0	8.0	13.0	**78.0**
14	1.0	0.0	**48.0**	0.0	0.0	29.0	13.0	9.0
15	0.0	2.0	18.0	0.0	0.0	**51.0**	1.0	28.0
16	3.0	9.0	5.0	0.0	0.0	20.0	**24.0**	**39.0**
17	0.0	3.0	36.0	0.0	0.0	**55.0**	4.0	2.0

Numerical value in bold red font indicates the proportion of sperm exhibiting a predominant pattern of PLCζ in sperm from each individual patient classified according to their clusters. Bold blue font indicates the proportion of sperm devoid PLCζ. Data are shown as percentages.

In the patient group the predominant PLCζ patterns frequently observed in sperm were either A+E+P or P+E that were classified according to Cluster 2 and Cluster 3 respectively (**Table 4.10**). Sperm from patients 6, 7 and 14 exhibited a single prominent pattern of PLCζ, which was localised to the E region as classified into Cluster 1. The total levels and proportion of sperm exhibiting PLCζ in patients 4 and 6 were significantly lower compared to all other patients. PLCζ was localised in the A+P and E region in some sperm from patients 4 and 6. In addition to patients 4 and 6, patients 7, 10, 13 and 16 showed higher proportions of sperm that were devoid of PLCζ and were classified into Cluster 1 or Cluster 4.

4.4 Specific clinical case studies identified during routine PLCζ screening

Patients 6, 7, 8, and 9 were selected from the total number of patients (n=17) processed during this study, and are presented specifically in this section as special case studies due to their originality. An additional patient, an infertile man (patient PM), with globozoospermia is also referred to this section and was originally obtained from the Department of Reproductive Medicine at Ghent University (Belgium). The precise characteristics of these cases, including sperm morphology, oocyte activation ability, and total levels of PLCζ and proportions of sperm exhibiting PLCζ are presented and described individually in the following sections.

4.4.1 Case study #1: Patient 6 - The potential effect of vasectomy/vasectomy reversal upon sperm morphology and the total levels of PLCζ

Vasectomy is a surgical procedure implemented to provide prolonged reliable sterilization for men (Belleannee *et al.*, 2013). Although, vasectomy was not designed to be reversible, men undergoing this procedure are able restore their fertility (Mui *et al*., 2014) by vasectomy reversal or infertility treatment via IVF or ICSI using surgically-retrieved sperm (Borges Junior *et al.*, 2003). Worryingly, vasectomy reversal is often non-reversible and does not necessarily restore fertility. Although, successful reversible surgeries are able to restore 90% of duct patency, the pregnancy rate falls only within a narrow range of 15%-50% (Feber & Ruiz, 1999; van Dongen *et al.*, 2012). The poor pregnancy outcome following successful vasectomy reversal is frequently associated with granuloma formation, inflammation, (Shapiro & Silber, 1979; Christiansen & Sandlow, 2003) and the production of anti-sperm antibodies (Carbone *et al.*, 1998). Moreover, vasectomy reversal is known to alter molecular and biochemical mechanisms within the epididymis (Legare *et al.*, 2010; Sullivan *et al.*, 2011), which may adversely affect the male reproductive tract.

While the effect of vasectomy surgery upon fertilisation outcome and pregnancy rate is clearly evident, nothing is known of how this procedure might affect sperm morphology or PLCζ expression. Acquisition of this information would be highly valuable as it may allow further understanding of whether this surgical procedure could detrimentally affect fertilisation ability.

The following case study describes PLCζ screening results from a male undergoing vasectomy reversal. Patient 6 was recruited from the ACU, Dundee. He was 39 years of age at the time of recruitment, had undergone a vasectomy reversal procedure, and was seeking infertility treatment at the ACU where he encountered recurrent ICSI failure. Patient 6 had fathered two children via natural conception from his previous relationship prior to vasectomy. Sperm from patient 6 were analysed for PLCζ fluorescence as described in **Section 4.2.3** and compared with control 1 (refer to **Table 4.1** and **Table 4.2** for control and patient details). Several interesting outcomes were revealed.

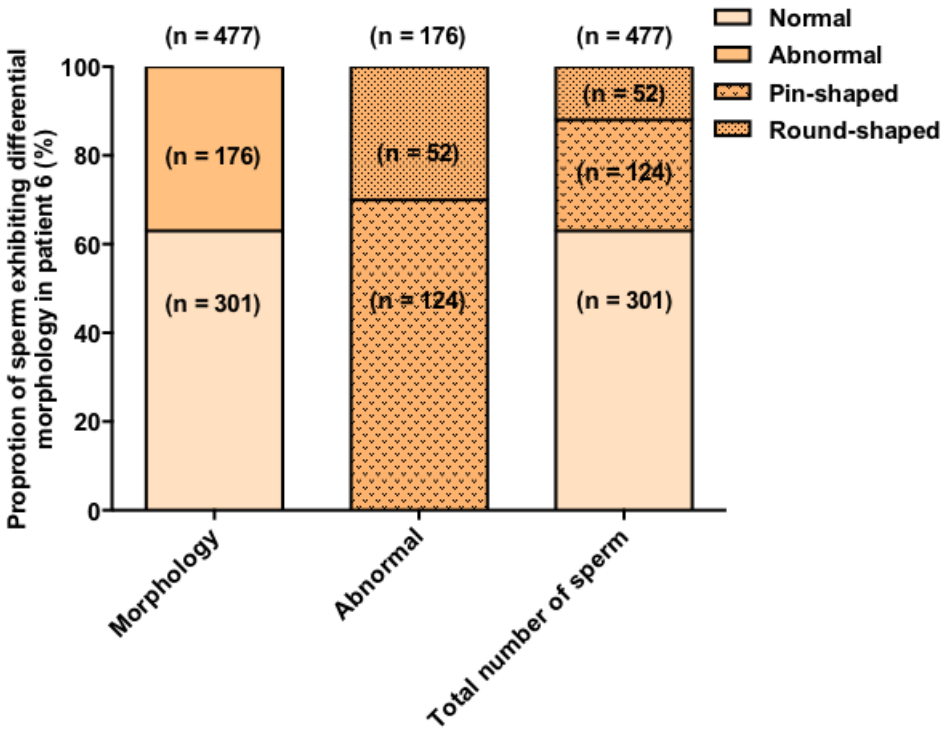


Figure 4.9: Proportion of sperm exhibiting normal and abnormal morphology in a total number of 477 sperm analysed from patient 6. The numerical value in the histograms represents the number of sperm analysed. Data are shown as percentages.

A total of 477 sperm from patient 6 were analysed (**Figure 4.9**) in order to quantify total PLCζ immunofluorescence levels and the proportions of sperm exhibiting PLCζ. The first observation obtained following the analysis was that 176 out of 477 sperm (37%) analysed from this patient exhibited abnormal morphology, while the remaining 301 sperm (63%) were morphologically normal. Morphologically normal sperm exhibiting PLCζ from control 1 is presented in **Figure 4.10A**. Among the morphologically abnormal sperm identified in patient 6, 70% had a pin-shaped head while 30% exhibited a round-shaped head (**Figure 4.9** and **4.10B**). Mean total fluorescence level of PLCζ in both morphological normal and abnormal sperm from patient 6 was 11.6 ± 0.2AU (**Figure 4.11**). Compared to control 1 (54.0 ± 0.9AU), the mean total levels of PLCζ in sperm from patient 6 (11.6 ± 0.2AU) were significant lower ($P \leq 0.05$; as indicated by the Student's *t*-test) (**Figure 4.12**).

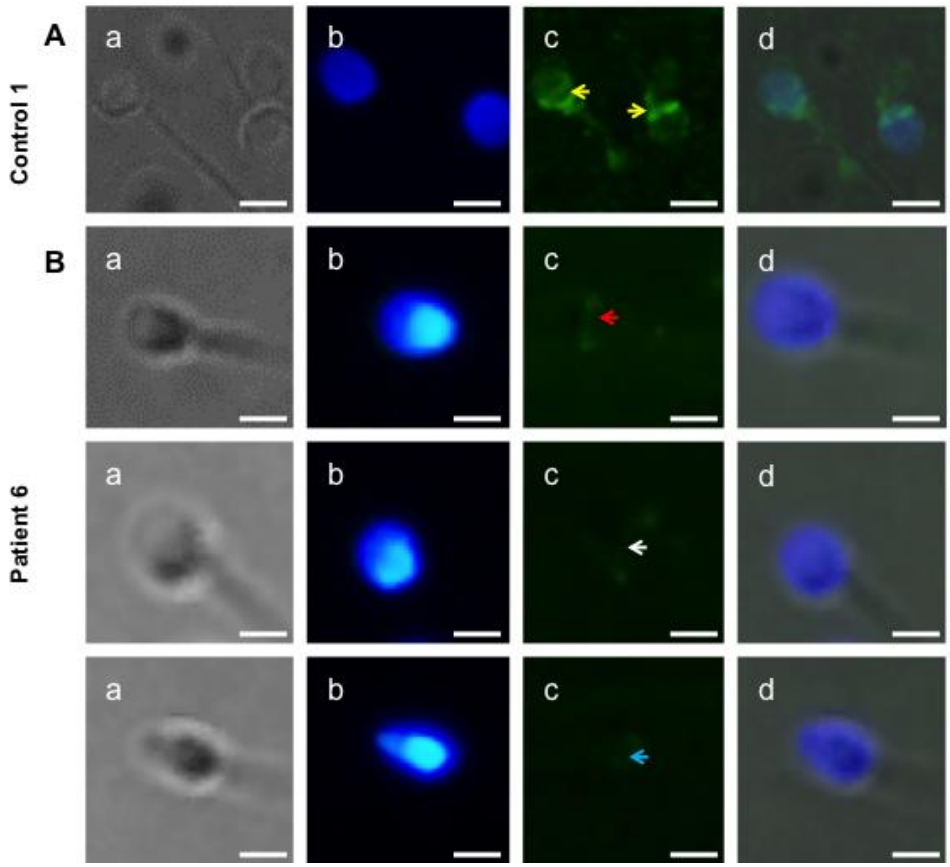


Figure 4.10: Representative microscopic images of (a) bright field, (b) nuclei, (c) phospholipase C zeta (PLCζ) and (d) overlay showing localisation of PLCζ in (**A**) sperm from control 1; Yellow arrow indicates PLCζ in equatorial and post-acrosomal regions of the sperm, and (**B**) sperm from patient 6; Arrows indicates PLCζ localisation in the equatorial region of morphologically normal (red arrow), round-shaped head (white arrow) and pin-shaped head (blue arrow) sperm. White scale bar indicates 5.0µm.

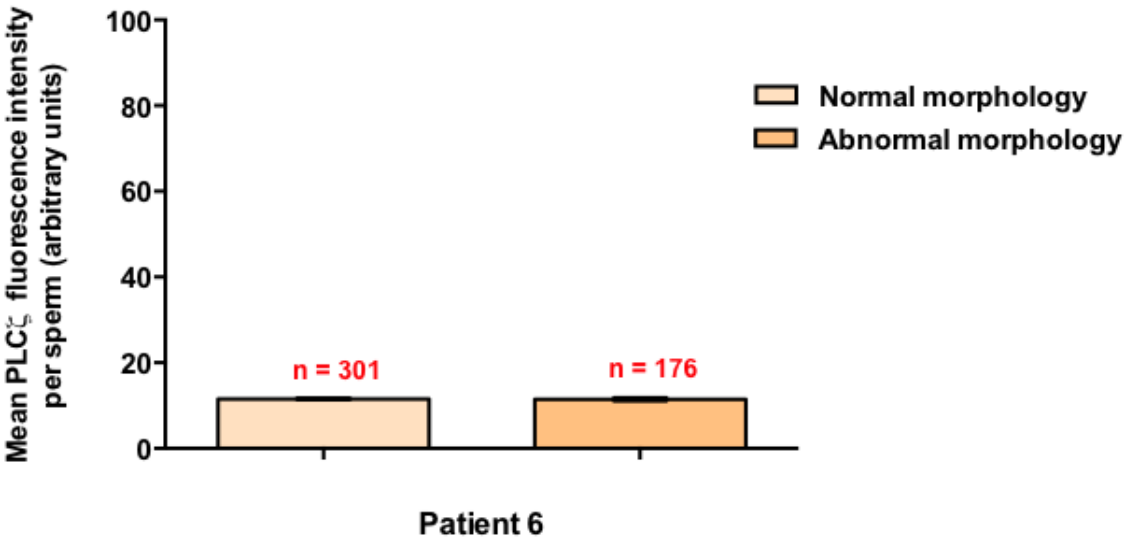


Figure 4.11: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in sperm from patient 6 with normal and abnormal morphology. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. There was no statistically significant difference in the total levels of PLCζ between morphological normal and abnormal sperm. The numerical values denoted in red font on each histogram represents the total number of sperm analysed. Data are shown as mean ± SEM.

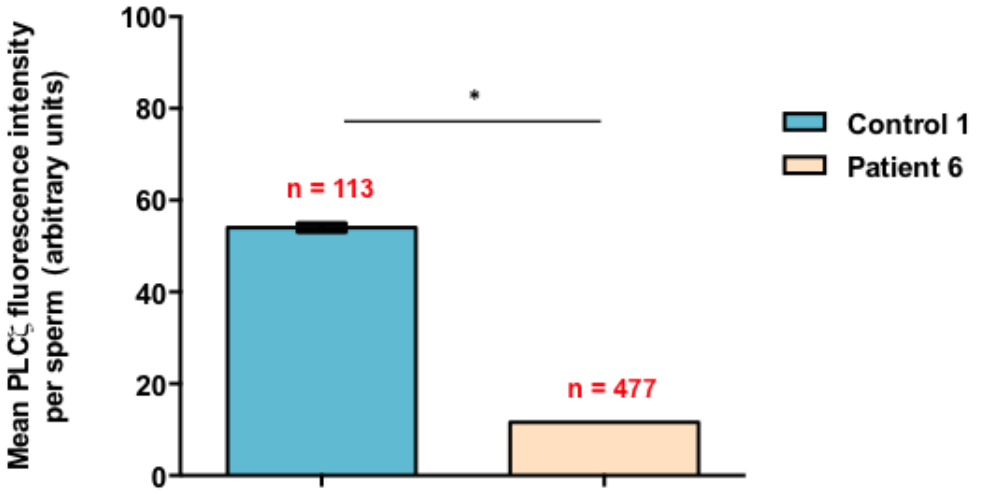


Figure 4.12: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in sperm from patient 6 as compared with sperm from control 1. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisk (*****) denotes a statistically significant difference ($P \leq 0.05$). The numerical values denoted in red font on each histogram represents the total number of sperm analysed. Data are shown as mean ± SEM.

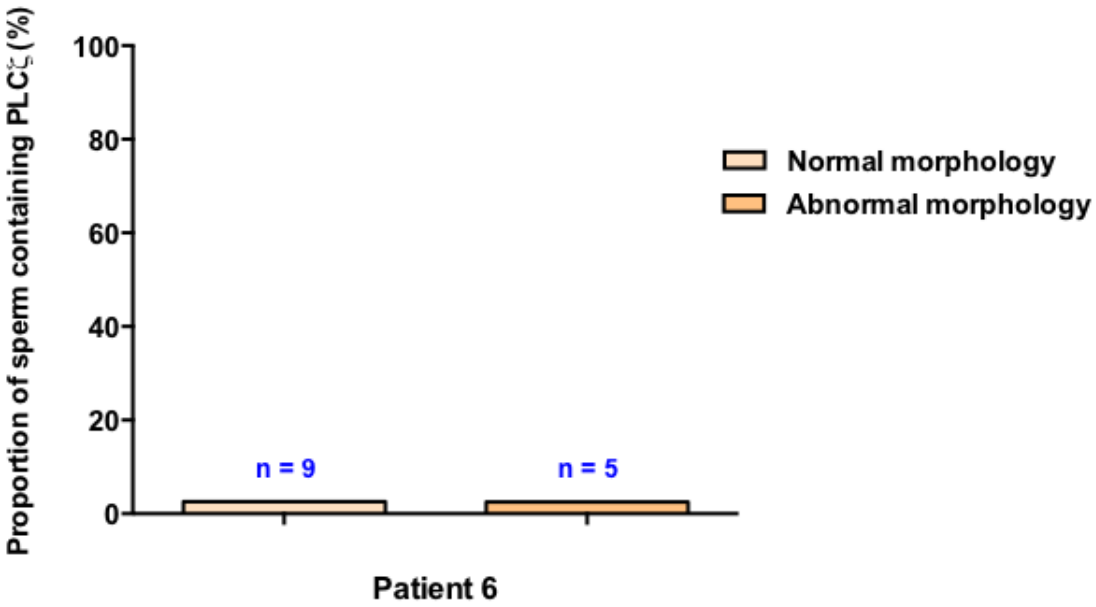


Figure 4.13: Mean proportion of morphologically normal and abnormal sperm from patient 6 exhibiting phospholipase C zeta (PLCζ). The proportion of sperm with PLCζ was determined with the use of ImageJ software. There were no statistically significant differences in the number of sperm exhibiting PLCζ between morphologically normal and abnormal sperm. The numerical values denoted in blue font on each histogram represents the number of sperm containing PLCζ out of the total number of sperm analysed. Data are shown as percentages.

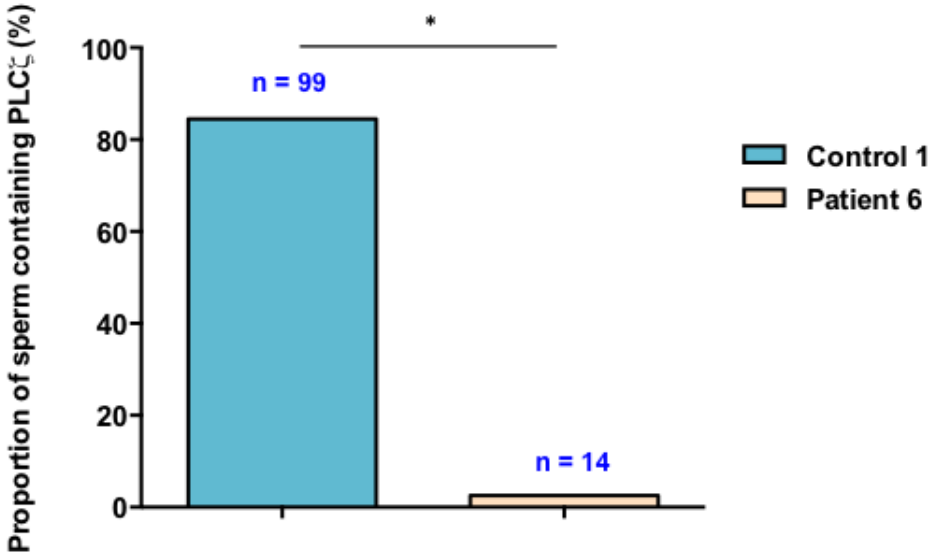


Figure 4.14: Mean proportion of sperm containing phospholipase C zeta (PLCζ) from patient 6 in comparison to sperm from control 1. The proportion of sperm with PLCζ was determined with the use of ImageJ software. Asterisk (*****) denotes a statistically significant difference ($P \leq 0.05$). The numerical values denoted in blue font on each histogram represents the number of sperm containing PLCζ out of the total number of sperm analysed. Data are shown as percentages.

Proportion analysis indicated that only 3% of sperm with normal and abnormal morphology from patient 6 showed the presence of PLCζ (**Figure 4.13**). Compared to control 1, sperm from patient 6 appeared to exhibit a punctuated pattern of PLCζ and reduced fluorescence intensity of PLCζ, which was localised to the equatorial region of the sperm head of normal, pin-shaped and round-headed sperm (**Figure 4.10B**). Comparative analysis of the proportion of sperm exhibiting PLCζ between patient 6 and control 1 showed a significant reduction ($P \leq 0.05$; as indicated by Fisher's Exact test) in patient 6 compared to control 1 (**Figure 4.14**). To our knowledge, this is the first study to demonstrate deficiency in the total level of PLCζ and proportion of sperm containing PLCζ in sperm from a vasectomy reversal patient. In addition, we identified the presence of morphological abnormalities in 30% of the sperm from this patient.

4.4.2 Case study #2: Patient 7 – Successful fertilisation following the application of AOA in a PLCζ-deficient patient and identification of a single nucleotide substitution in the PLCζ promoter region

Although, the success of ICSI is currently accountable for fertilisation rates of approximately 80% during ART, fertilisation failure still occurs following ICSI in some patients and OAD is postulated to be the primary factor responsible (Vanden Meerschaut *et al.*, 2014). The application of AOA in combination with ICSI is frequently used in OAD cases, and successful fertilisation following such treatment has been widely reported (Heindryckx *et al.*, 2008). Various studies have demonstrated PLCζ deficiency in sperm from infertile men diagnosed with OAD, which clearly suggests the fundamental role of PLCζ for oocyte activation (Kashir *et al.*, 2013b). In addition, as demonstrated by previous studies, there is also the possibility that OAD in some patients may be due to mutation of the PLCζ gene, which could compromise the oocyte activation ability of such sperm by altering

expression level or compromising the structure of the protein (Heytens *et al.*, 2009; Kashir *et al.*, 2012b).

The following case study relates to ICSI failure and links PLCζ deficiency and OAD with genetic mutation. This case study also describes the first application of AOA in a Scottish fertility unit. Case study 2 refers to patient 7, who encountered total fertilisation failure (TFF) following an initial 1st ICSI cycle. In addition, immunofluorescent analysis of the sperm from this patient revealed significant deficiency in the total levels of PLCζ. Microscopic imaging showed that, out of 100 sperm analysed, 18% exhibited morphological abnormality (pin-headed sperm). The remaining 82% of sperm showed normal morphology, similar to control 1 (**Figure 4.15A**). From the total number of sperm analysed, 46% of the sperm from patient 7 (43% normal morphology and 3% pin-shaped head sperm) exhibited PLCζ localisation in the equatorial region of the sperm head. PLCζ localisation exhibited punctuated patterning in the equatorial region in 11% of sperm (**Figure 4.15B**). Immunofluorescence analysis performed on sperm from patient 7 indicated that the total levels of PLCζ (27.14 ± 0.49AU) (**Figure 4.16**) and the proportion of sperm exhibiting PLCζ were significantly reduced ($P \leq 0.05$; as indicated by the Student's *t*-test) and ($P \leq 0.05$; as indicated by Fisher's Exact test) compared to sperm from control 1 (**Figure 4.17**). The total immunofluorescence levels of PLCζ, and the proportions of sperm containing PLCζ, in sperm from patient 7 were reduced by 49% and 41% respectively, compared to control 1.

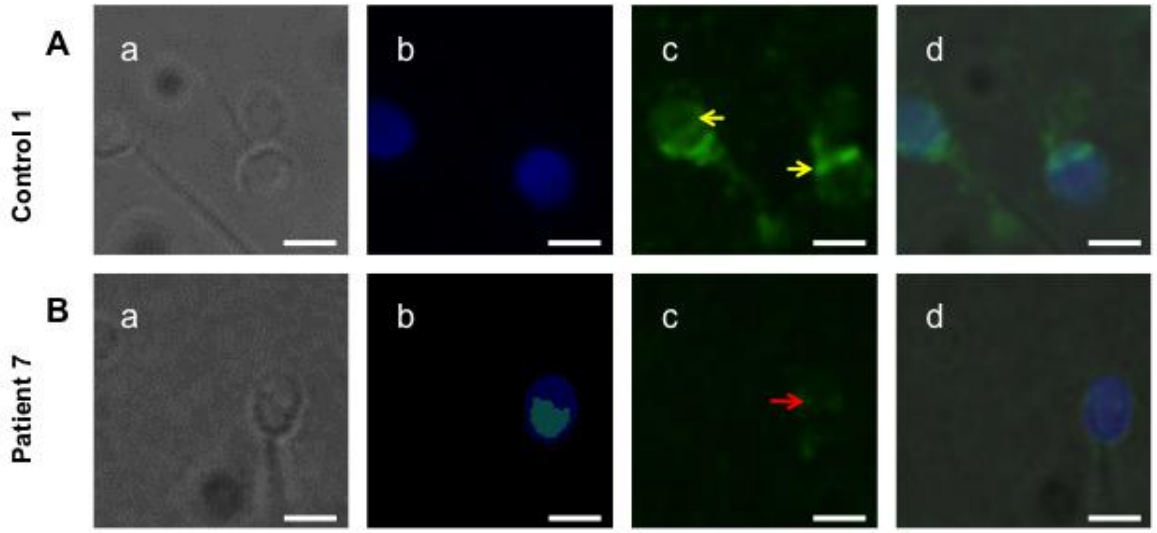


Figure 4.15: Representative microscopic images of (a) bright field, (b) nuclei, (c) phospholipase C zeta (PLCζ) and (d) overlay showing localisation of PLCζ in (**A**) sperm from control 1; Yellow arrow indicates PLCζ in equatorial and post-acrosomal regions of the sperm, and (**B**) sperm from patient 7; Red arrow indicates reduced fluorescence intensity levels of PLCζ in equatorial region of the sperm. White scale bar indicates 5.0µm.

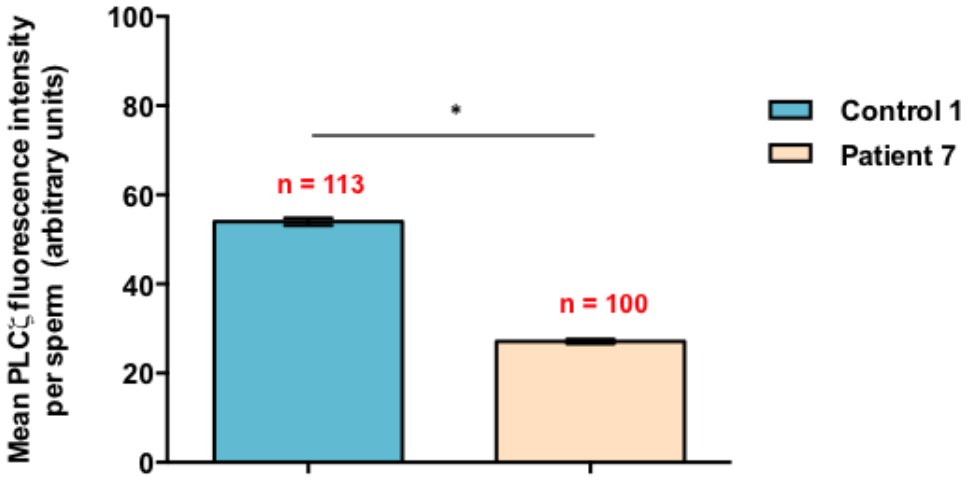


Figure 4.16: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in sperm from patient 7 as compared with sperm from control 1. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisk (*****) denotes a statistically significant difference ($P \leq 0.05$). The numerical values denoted in red font on each histogram represents the total number of sperm analysed. Data are shown as mean ± SEM.

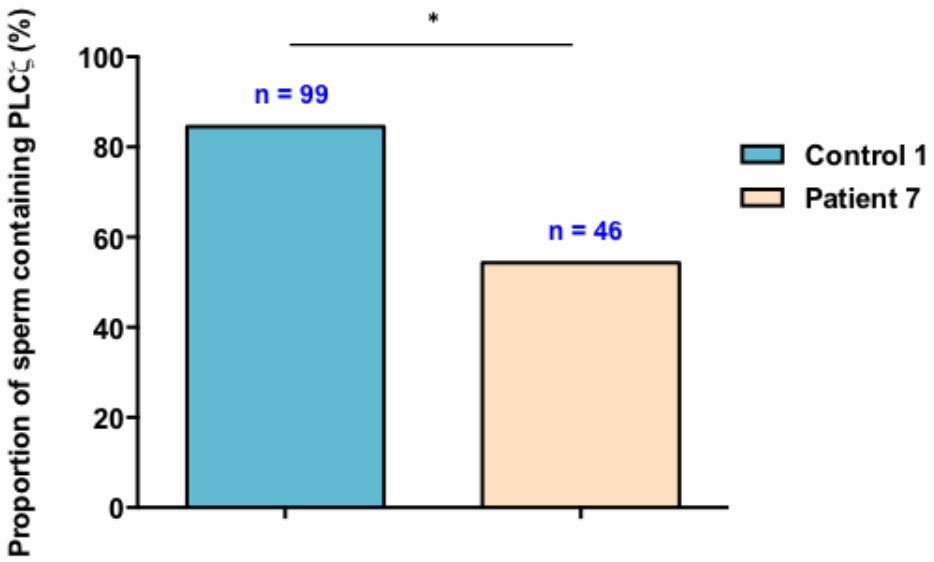


Figure 4.17: Mean proportion of sperm containing phospholipase C zeta (PLCζ) from patient 7 in comparison to sperm from control 1. The number of sperm exhibiting PLCζ was determined with the use of ImageJ software. Asterisk (*****) denotes a statistically significant difference ($P \leq 0.05$). The numerical values denoted in blue font on each histogram represents the number of sperm containing PLCζ out of the total number of sperm analysed. Data are shown as percentages.

Following dissemination of these results to the ACU, the clinical team attempted their first AOA treatment during a 2nd ICSI cycle, and attained successful fertilisation. From a total of 11 eggs, 6 eggs were fertilised (54% of fertilisation rate) and two embryos were selected. One was implanted and one was frozen. However, subsequent information from the clinic advised that pregnancy was not sustained.

Routine genetic screening analysis performed on patient 7 carried out by Miss Siti Nornadhirah Amdani in a separate research project additionally revealed a SNP in the promoter of this patient is PLCζ gene at position +109C > A (forward) −263G > T (reverse) (**Figure 4.18**). This SNP is known and is clearly shown in genetic databases (Personal Communication, Miss Siti Nornadhirah Amdani). The genetic information retrieved from University of California, Santa Cruz (UCSC) is presented and described in **Appendix 2.0**.

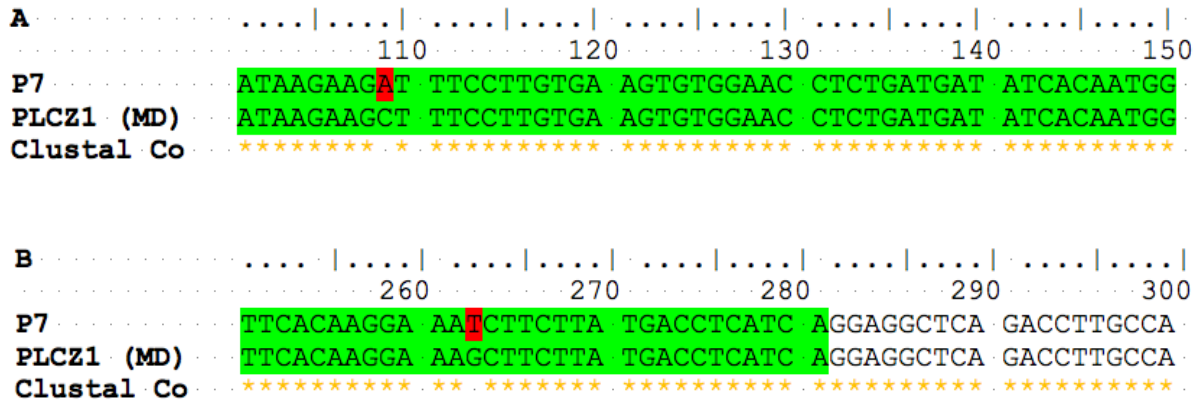


Figure 4.18: Multiple sequence alignment (ClustalW) of patient 7's phospholipase C zeta (PLCζ) promoter-exon 1 (**A**) forward and (**B**) reverse sequences with human PLCζ1 sequence from Mutation discovery. Gold asterisks (*****) denote identical nucleotides in aligned sequences. The green highlighted region indicates the PLCζ promoter region. The red highlighted region indicates the nucleotide base change.

The promoter region of a gene is responsible for initiating transcription and thus plays a critical regulation role in gene expression. The SNP identified in the PLCζ gene promoter region of patient 7 could therefore change the expression of PLCζ leading to the deficiency of PLCζ seen in this patient. However, the precise mechanism underlying this condition has yet be established and was beyond the scope of this thesis. However, interestingly, the same SNP was detected in other case studies reported here (See **Section 4.4.4**).

4.4.3 Case study #3: Patients 8 and 9 – Could the dysfunction of a potential unknown oocyte factor underlie ICSI failure in patients with OAD but exhibiting normal levels of PLCζ?

Recent reports strongly suggest the probable involvement of an as yet unidentified oocyte-borne factor localised in small vesicles within the ooplasm, which binds and activates PLCζ in order to generate Ca^{2+} oscillations by hydrolysing PLCζ in close proximity to its PIP_2 substrate (Yu *et al.*, 2012; Swann & Lai, 2013) (**See Chapter 1-Figure 1.13**). Such findings may explain the presence of inactive PLCζ in sperm which in turn attain functional capability only following gamete fusion (Nomikos *et al.*, 2013b). While researchers are

focusing upon identifying this potential oocyte factor, it would be interesting to encounter cases of TFF following ICSI, in which the male exhibited comparable levels of PLCζ with fertile men and did not exhibit mutation of the PLCζ gene. It remains undetermined whether such conditions might be associated with deficiency or abnormality of the unknown oocyte factor, which in turn may inhibit the activation of PLCζ and thus prevent oocyte activation.

During the course of the present study, we identified twelve patients who had encountered TFF following ICSI (**Table 4.2**) and (**Table 4.13**). Here, we represent two different examples: Patients 8 and 9. Patient 8 underwent a first IVF cycle and achieved only an 18% fertilisation rate. In his 2nd cycle of ICSI treatment, he experienced TFF with 0% of oocytes fertilised from a total number of 7 oocytes retrieved. Semen analysis showed normal sperm parameters and morphology. There were no indications of an oocyte problem based on current screening methods. The first infertility treatment for patient 9 was IVF and resulted in 0% fertilisation from a total of 4 oocytes retrieved. Cycle 2, involving ICSI treatment, also yielded 0% fertilisation. Since clinical opinion suggested that the poor fertilisation rate of patient 9 could be due to oocyte deficiency, the 3rd cycle of ICSI was performed with oocytes from a donor with proven fertility. From a total number of 11 oocytes, only 1 oocyte was fertilised. This might suggest therefore that the most likely problem related to a sperm deficiency, and it was interesting to note that the embryo arising from the one fertilised oocyte was of poor quality and following transfer, failed to achieve pregnancy.

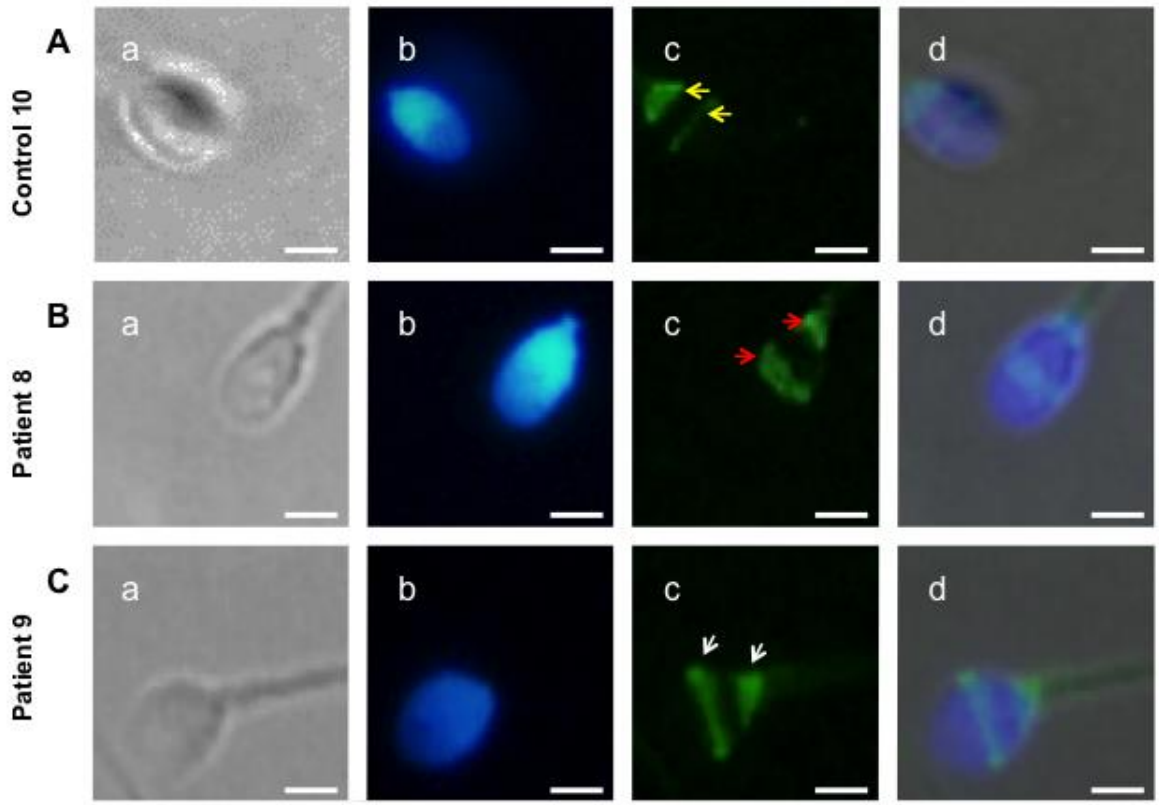


Figure 4.19: Representative microscopic images of (a) bright field, (b) nuclei, (c) phospholipase C zeta (PLCζ) and (d) overlay showing localisation of PLCζ in sperm from (**A**) control 10; yellow arrows (**B**) patient 8; red arrows and (**C**) Patient 9; white arrows, indicating PLCζ in equatorial and post-acrosomal regions of the sperm. White scale bar indicates 5.0µm.

PLCζ immunocytochemistry analysis was performed in sperm from patients 8 and 9 in comparison to control 10. PLCζ patterning was predominantly localised in the P+E regions of sperm from patient 8 and 9 (**Table 4.9**), which was comparable to control 10 (**Table 4.8**), (**Figure 4.19**). The total levels of PLCζ from patient 8 and 9 were 37.67 ± 1.03AU and 47.20 ± 0.63AU respectively but were not significantly ($P \geq 0.05$; as indicated by One-Way ANOVA) different from the control (34.15 ± 0.95AU) (**Figure 4.20**). Similarly, proportional analysis of sperm exhibiting PLCζ in sperm from patient 8 (97%) and 9 (97%) were similar to the control (96%) and did not show statistically significant differences (**Figure 4.21**).

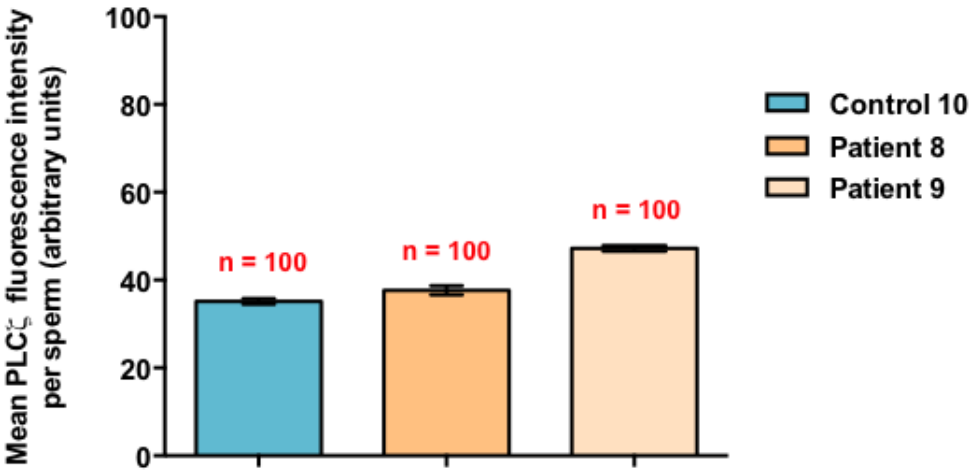


Figure 4.20: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in sperm from patients 8 and 9 as compared with sperm from control 10. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. There were no statistically significant differences in the total levels of PLCζ between control and patients. The numerical values denoted in red font on each histogram represents the total number of sperm analysed. Data are shown as mean ± SEM.

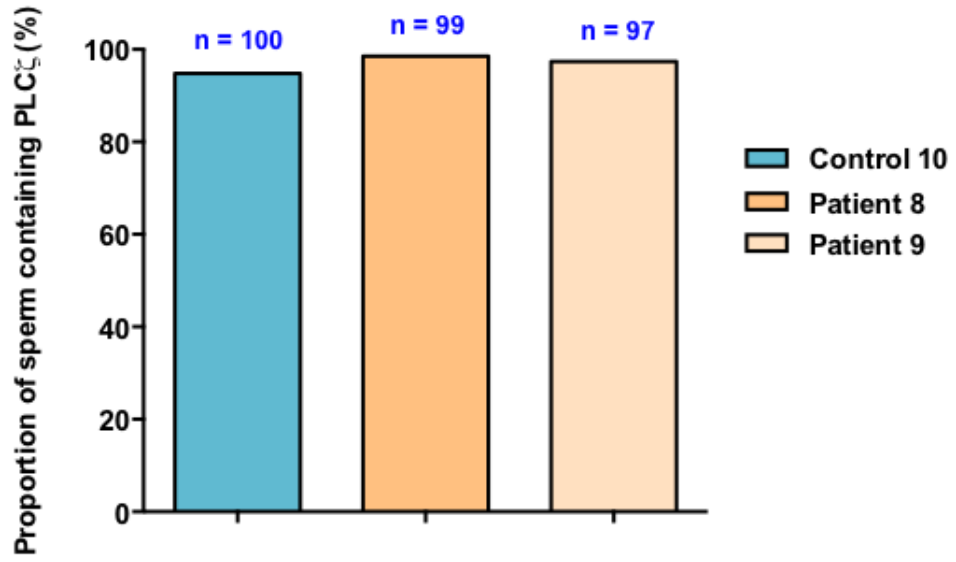


Figure 4.21: Mean proportion of sperm containing phospholipase C zeta (PLCζ) from patients 8 and 9 in comparison to sperm from control 10. The number of sperm exhibiting PLCζ was determined with the use of ImageJ software. There were no statistically significant differences in the number of sperm exhibiting PLCζ between control and patients. The numerical values denoted in blue font on each histogram represents the number of sperm containing PLCζ out of the total number of sperm analysed. Data are shown as percentages.

Although patient 8 and 9 encountered significant ICSI failure, sperm from these patients did not show deficiency in the total levels of PLCζ. Moreover, the levels and localisation of PLCζ were comparable with sperm from control 10. These findings may suggest that some ICSI failure cases may not be related to PLCζ, but rather a potential oocyte factor required for the successful activation of PLCζ. However, in the case of patient 9, since donor oocytes also exhibited low fertilisation. This may imply a PLCζ problem that was not identified by our current immunofluorescent or genetic test and is discussed further later in this chapter.

4.4.4 Case study # 4: Patient PM - Deficiency and novel localisation of PLCζ in a globozoospermic patient and a possible link to an SNP in the PLCζ promoter

Globozoospermia is a sperm morphology abnormality in which the sperm head exhibits a round head due to the lack of an acrosome. This condition is encountered in 0.1% of infertile men (Dam *et al.*, 2007a). The fertilisation rate of globozoospermic men is markedly low following ICSI and thus frequently treated with AOA. The application of AOA in such a severe condition is highly beneficial in order to achieve successful fertilisation by restoring fertilisation rates (Nasr-Esfahani *et al.*, 2010), especially in globozoospermic patients (Taylor *et al.*, 2010). A clear association between PLCζ deficiency and globozoospermic cases with OAD has been demonstrated by several studies (Heytens *et al.*, 2009; Kashir *et al.*, 2012c; Lee *et al.*, 2014). While, genetic mutation in the PLCζ gene of an OAD non-globozoospermic patient has been reported previously (Heytens *et al.*, 2009), it is essential to identify whether mutations of PLCζ may exist in globozoospermic patients, especially considering that mutation of two genes, *DPYI9L2* and *SPATAI6*, have already been

linked to globozoospermia (Kuentz *et al.*, 2013). As yet, there have been no reports linking globozoospermia with PLCζ mutation.

The following study describes the case of a globozoospermic infertile man. A semen sample from patient PM was recruited from Ghent University Hospital, Belgium. The clinical characteristics, history of infertility treatment, and a brief outcome of PLCζ analysis are tabulated (**Table 4.11**).

Table 4.11: Clinical characteristics, a case history and ART treatment outcome of patient PM.

Patient PM	**Sperm analysis Motile sperm count (%)**				**Morphology (%)**	**Concentration (M/ml)**	**Proportion of sperm [+PLCζ] (%)**
Age	**a**	**b**	**c**	**d**			
43	0	16	3	81	100% (Globozoospermic)	10	8
IVF cycles					**ART treatments**	**Fertilisation rates**	**ART outcomes**
Cycle 1					ICSI + AOA	4/6 (67%)	Miscarriage
Cycle 2					ICSI + AOA	3/5 (60%)	N/A
Cycle 3					ICSI + AOA	4/5 (80%)	Singleton delivery

[+PLCζ] = Total number of sperm exhibiting PLCζ is
N/A = Not available

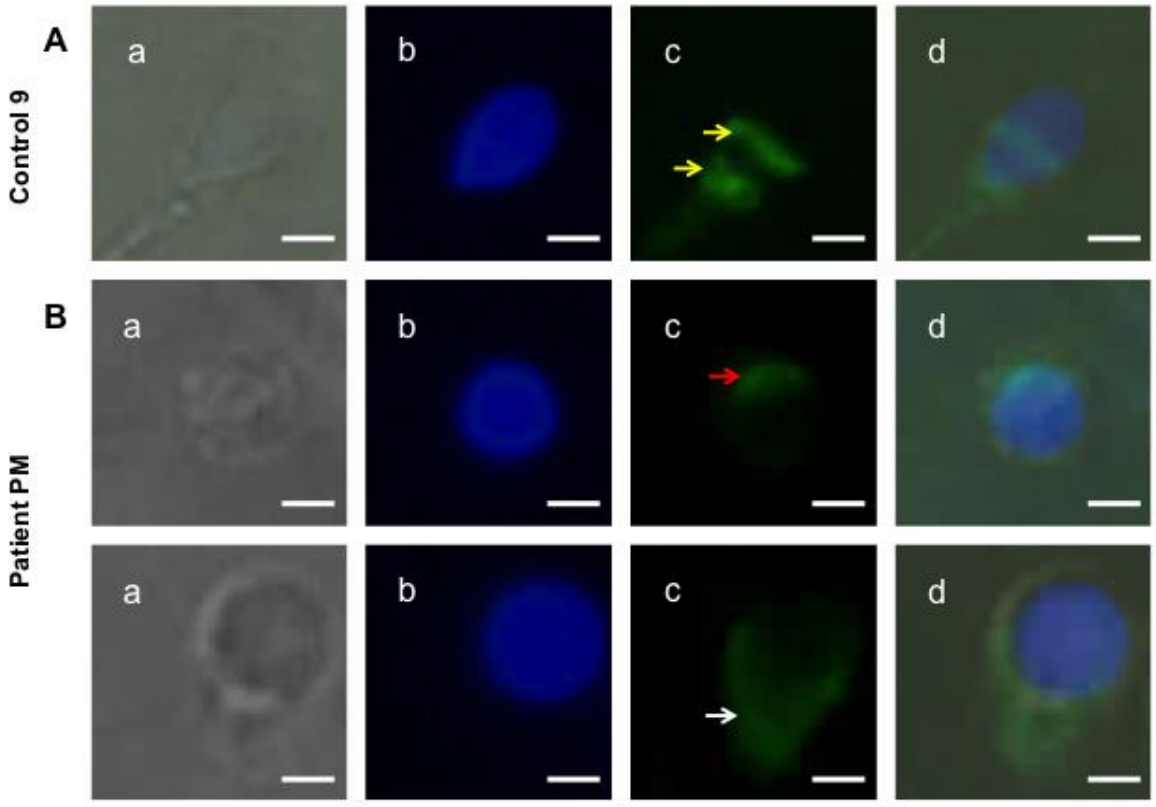


Figure 4.22: Representative microscopic images of (a) bright field, (b) nuclei, (c) phospholipase C zeta (PLCζ) and (d) overlay showing localisation of PLCζ in sperm from (**A**) control 9; yellow arrow indicates PLCζ in equatorial and post-acrosomal regions of the sperm, and (**B**) patient PM; red and white arrow indicates anterior and mid-piece localisation of PLCζ . White scale bar indicates 5.0µm.

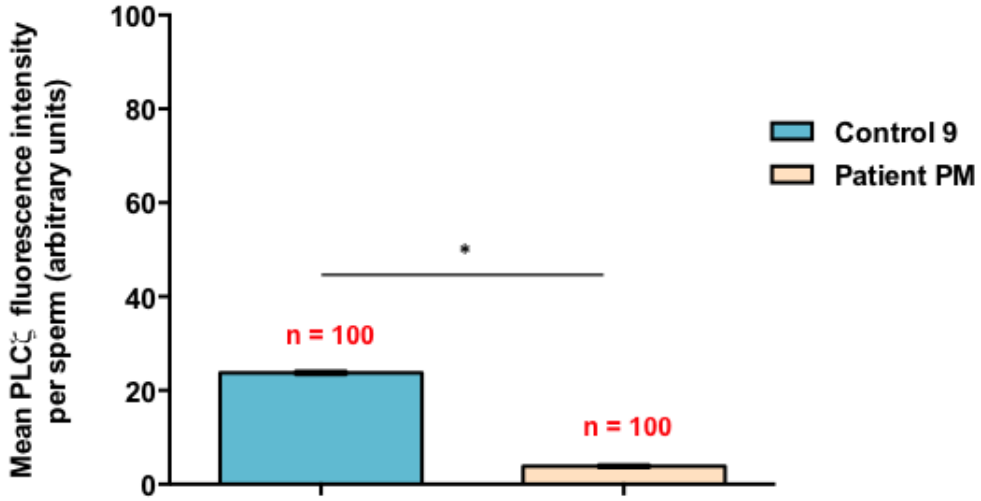


Figure 4.23: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in sperm from patient PM as compared with sperm from control 9. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisk (*) denotes a statistically significant difference ($P \leq 0.05$). The numerical values denoted in red font on each histogram represents the total number of sperm analysed. Data are shown as mean ± SEM.

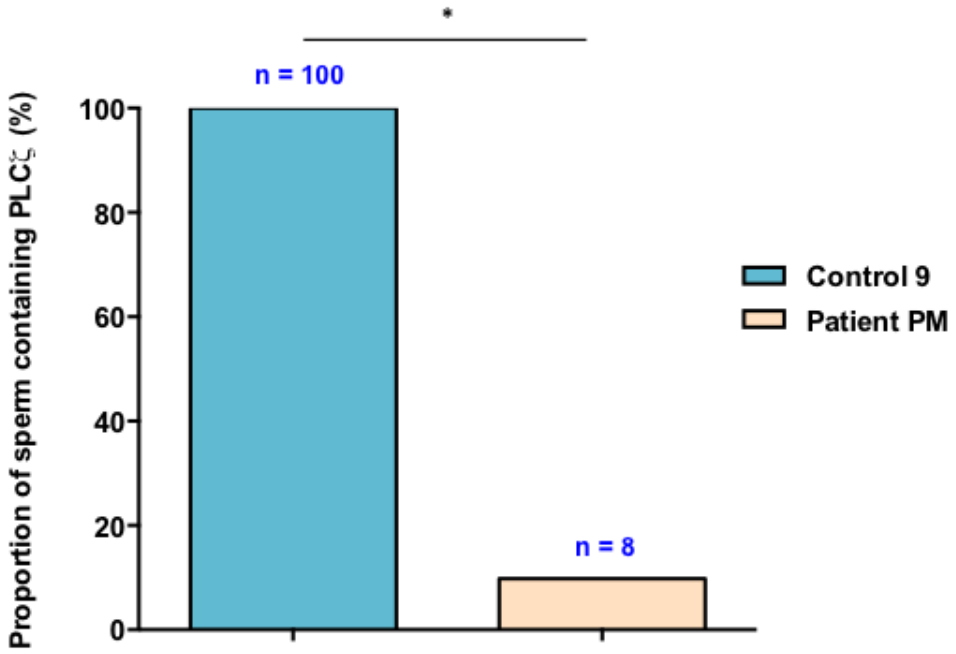


Figure 4.24: Mean proportion of sperm containing phospholipase C zeta (PLCζ) from patient PM in comparison to sperm from control 9. The number of sperm with PLCζ was determined with the use of ImageJ software. Asterisk (*) denotes a statisitcally significant difference ($P \leq 0.05$). The numerical values denoted in blue font on each histogram represents the number of sperm containing PLCζ out of the total number of sperm analysed. Data are shown as percentages.

PLCζ immunofluorescence analysis of this patient was compared with sperm from control 9. In addition, the patient sample was also subjected to genetic screening of the PLCζ promoter region (carried out by Miss Siti Nornadhirah Amdani). The total immunofluorescence levels of PLCζ in patient PM (3.82 ± 0.26AU) were significantly lower ($P \leq 0.05$; as indicated by the Student's *t*-test) than sperm from control 9 (23.7 ± 0.32AU) (**Figure 4.23**). Proportional analysis further indicated that the number of sperm containing PLCζ from patient PM were also significantly lower in comparison to control 9 ($P \leq 0.05$; as indicated by Fisher's Exact test) (**Figure 4.24**). From a total number of 100 sperm analysed from patient PM, only 8 sperm showed the presence of PLCζ, which was predominantly localised within the mid-piece of the sperm, as indicated by the white arrow (**Figure 4.22B**). In addition, localisation pattern of PLCζ in the anterior position within the sperm head is shown with a red arrow (**Figure 4.22B**) and was observed in 1 out of 100 sperm analysed. As

with patient 7, genetic screening identified a SNP in the PLCζ gene promoter region at position +109C > A (forward) −263G > T (reverse) (**Figure 4.25**). This is interesting for patient PM as the PLCζ promoter is bi-directional and shared with another gene (CAPZA3) which is responsible for sperm morphology and architecture (Geyer *et al.*, 2009; Coward *et al.*, 2011).

Figure 4.25: Multiple sequence alignment (ClustalW) of patient PM's phospholipase C zeta (PLCζ) promoter-exon 1 (**A**) forward and (**B**) reverse sequences with human PLCζ1 sequence from Mutation discovery. Gold asterisks (*****) denote identical nucleotides in aligned sequences. The green highlighted region indicates PLCζ promoter region within the sequence. The red highlighted region indicates the nucleotide base change.

The SNP identified in patient PM, a globozoospermic patient, may raise a question as to whether the morphological abnormalities in this patient could be attributed to this SNP. The detrimental effect of such a SNP upon the expression of CAPZA3, and if this may have led to the globozoospemic condition seen in this patient, remains unknown and is difficult to ascertain. Nevertheless, it is very interesting that both patient PM and patient 7 (Case study #2) possessed the same SNP in the bi-directional promoter of PLCζ. It is even more curious that one of these patients was globozoospermic and one was not. Whether the SNP identified here plays a functional role, however, is difficult to prove and was beyond the remit of this thesis.

4.5 Development of a novel PLCζ scoring system to predict oocyte activation deficiency and to compare fertilisation outcome following ART

Here cluster classification data arising from **Section 4.3.2** and **Table 4.6** was utilised to develop a novel scoring system for the total level of PLCζ in human sperm, which may, ultimately allow the utilisation of PLCζ immunofluorescence screening as a clinical tool to predict fertilisation capacity and assess risk for oocyte activation deficiency (**Table 4.12**).

As described in **Section 4.3.2**, Clusters 1, 2 and 4 represent high, intermediate and low levels of PLCζ and proportion of sperm exhibiting PLCζ respectively. Cluster 3 represents a unique classification indicating sperm exhibiting low immunofluorescence levels and but with higher a proportion of sperm containing PLCζ. Column A (**Table 4.12)** describes a new PLCζ scoring system developed based upon the mean total levels of PLCζ and proportion of sperm exhibiting PLCζ obtained from the clusters created in **Section 4.3.2** and **Table 4.6**. The number of controls and patients whose total levels of PLCζ fell within their specific scoring ranges were tabulated. The data collectively indicated that the majority of controls (46%) and patients (41%) exhibited total levels of PLCζ within the low + high scoring board (**#**), which represents low levels of PLCζ exhibiting higher proportion of sperm containing PLCζ.

Column B (**Table 4.12)** specifies the ART treatments associated with each of the samples. Column C, D and E (**Table 4.12**) indicate specific case studies of patients identified during this study with their respective PLCζ score. The detailed clinical characteristics and research analysis arising from these case study patients are presented specifically in previous **Section 4.4**.

Table 4.12: Phospholipase C zeta (PLCζ) scoring system developed based upon mean total levels of PLCζ and proportion of sperm exhibiting PLCζ in sperm from 30 individual males.

(A) PLCζ scoring system			Controls (n=13)	Patients (n=17)
Cluster 1	High	***	3	4
Cluster 2	Intermediate	**	3	4
Cluster 4	Low	*	1	2
Cluster 3	Low+high	#	6	7

(B) Number of controls and patients sub-divided according to their infertility treatments and characteristics of PLCζ.

PLCζ scoring		***	**	*	#		***	**	*	#
ART treatment:	Total (n)					Total (n)				
IVF	11	3	3	1	4	5	1	1	-	3
ICSI	2	-	-	-	2	12	3	3	2	4

(C) Vasectomy reversal patient. Exhibited sperm morphology abnormalities (Section 4.4.1).

ICSI	-	-	1	* 1	

(D) Single Nucleotide Polymorphism (SNP) in the PLCζ gene promoter region (Section 4.4.2).

ICSI	-	-	1	** 1	

(E) Suspected to be an unknown oocyte factor deficiency, which may be inked with PLCζ activation (Section 4.4.3).

ICSI	-	-	2	*** 1	# 1

Asterisks (***, **, *) denote high, intermediate and low levels of PLCζ classified according to clusters. Hash mark (#) denotes low levels of PLCζ with a high proportion of sperm containing PLCζ in the sperm from Cluster 3.

From the total number of 30 males studied herein, 13 were controls and 17 were patients. The majority of controls (11 out of 13, 85%) were involved in IVF treatments, while the remaining 2 were treated by ICSI. Sperm from the males treated with IVF predominantly involved female or idiopathic factors (**Table 4.1**), and achieved either high (***) or low PLCζ levels with a higher proportion of sperm exhibiting PLCζ (#) scores with the new PLCζ scoring system. Fertilisation rates among 85% of the controls treated with IVF ranged from 33%-100% (**Table 4.13**). Controls 12 and 13, both involved in idiopathic cases of infertility, were treated with ICSI, due to very low motile sperm counts and poor progressive motility

(**Table 4.13**). These controls exhibited the low PLCζ immunofluorescence levels with higher proportion of sperm exhibiting PLCζ (#) scores. In contrast, control 3, who also exhibited a very low motile sperm count, was treated with IVF instead of ICSI. However, progressive sperm motility in this control was much higher (94%) than control 16 (45%). This may therefore justify the selection of IVF treatment for control 3. However, sperm from control 3 exhibited a very low (*) PLCζ score, which may explain the low fertilisation rates obtained. The PLCζ scoring system may therefore be able to predict fertilisation ability and could be useful in a clinical scenario to assess the likelihood of IVF or ICSI success.

As observed among the controls group, most patients (7 out of 17), exhibited a low PLCζ score (#), while the remaining patients fell within the high and intermediate scores (***, **). Ten patients (8 ICSI and 2 IVF patients) exhibited a 0% fertilisation rate or TFF. Of the total number of patients with a 0% fertilisation rate, 2 patients scored the lowest score in terms of PLCζ (*). The remaining 10 patients exhibiting TFF scored a wide variation in the PLCζ scoring system ranging from highest (***) to higher proportion of sperm exhibiting low levels of PLCζ (#). Among these patients, 3 patients scored higher proportion of sperm exhibiting low levels of PLCζ (#), suggesting that poor fertilisation outcome may be related to low PLCζ levels, although the proportion of sperm exhibiting PLCζ was higher as classified according to Cluster 3. However, 5 patients with high (***) and intermediate (**) PLCζ scores also exhibited TFF indicating that fertilisation failure among these patients may not be related to PLCζ, but could be due to poor sperm parameters, as some of the patients in this study exhibited very low motile sperm counts and poor progressive motility. Moreover, these patients were classified with unexplained infertility and this may therefore further indicate that unsuccessful fertilisation could be attributed to unknown male or female infertility factors associated with poor gamete quality, sperm/oocyte DNA fragmentation, genetic abnormalities or technical errors during ART procedure.

Table 4.13: Total level of phospholipase C zeta (PLCζ) based upon the scoring system tabulated for each control corresponding to their semen analysis ART treatment and fertilisation rates.

Controls (n=13)	Motile count (10^6 /mL)	Progressive motility (a+b) %	ART treatment IVF / ICSI	Number of oocytes fertilised (%)	PLCζ SCORING
1	13.8	81	IVF	6/7 (86)	***
2	10.6	72	IVF	2/2 (100)	#
3	6.3	94	IVF	6/12 (50)	*
4	32.4	81	IVF	1/1 (100)	**
5	16.2	95	IVF	11/14 (79)	**
6	16.0	62	IVF	1/1 (100)	#
7	19.8	79	IVF	6/6 (100)	**
8	20.3	81	IVF	2/6 (33)	***
9	12.9	86	IVF	4/7 (57)	***
10	15.5	87	IVF	2/5 (40)	#
11	41.0	91	IVF	2/2 (100)	#
12	6.1	54	ICSI	6/8 (75)	#
13	5.6	45	ICSI	4/5 (80)	#

Cluster 1 *** = High.
Cluster 2 ** = Intermediate.
Cluster 4 * = Low.
Cluster 3 # = Low levels of PLCζ with higher proportion of sperm exhibiting PLCζ.
Cluster indicating the classification of PLCζ.

Table 4.14: Total level of phospholipase C zeta (PLCζ) based upon the scoring system tabulated for each patient corresponding to their semen analysis ART treatment and fertilisation rates.

Patients (n=17)	Motile count (10^6 /mL)	Progressive motility (a+b) %	ART treatment IVF / ICSI	Number of oocytes fertilised (%)	PLCζ SCORING
1	25.0	41	ICSI	0/8 (0)	***
2	7.6	91	ICSI	13/13 (100)	#
3	4.2	53	ICSI	1/7 (14)	#
4	1.7	61	ICSI	0/4 (0)	*
5	10.4	69	ICSI	8/9 (89)	***
6	22.3	67	ICSI	0/10 (0)	*
7	3.1	38	ICSI	0/9 (0)	**
8	15.0	86	ICSI	0/7 (0)	#
9	5.5	72	IVF	1/11 (9)	***
10	44.4	48	ICSI	2/15 (13)	***
11	29.4	9	IVF	0/12 (0)	**
12	19.1	60	ICSI	1/7 (14)	#
13	13.9	76	IVF	2/20 (10)	#
14	3.3	47	ICSI	0/4 (0)	**
15	27.0	85	ICSI	0/6 (0)	#
16	6.7	71	IVF	0/6 (0)	#
17	33.4	69	ICSI	0/12 (0)	**

Cluster 1 *** = High.
Cluster 2 ** = Intermediate.
Cluster 4 * = Low.
Cluster 3 # = Low levels of PLCζ with higher proportion of sperm exhibiting PLCζ.
Cluster indicating the classification of PLCζ.

Current findings do appear to show a potential association between fertilisation rates and the total levels and proportion of sperm exhibiting PLCζ, however some controls and patients exhibiting TFF scored high (***) or intermediate (**) PLCζ scores. These findings firstly suggest that the high variability of PLCζ exhibited by sperm may exert influence over fertilisation rates and that this may also vary between fertile controls and infertile patients. Secondly, these data suggest that the oocyte itself may exert a certain degree of influence in response to receiving PLCζ following gamete fusion. Finally, the present data also indicate that poor fertilisation rate in those men with high or intermediate score of PLCζ might be due to poor oocyte/sperm quality and abnormal sperm parameters. Therefore, it is clear that when compared individually between each control and patient, the links between total levels and proportion of sperm exhibiting PLCζ with fertilisation rate may not be consistent and could be influenced by other unknown factors, thus contributing to poor fertilisation rate following ART treatment. Further correlation analysis between fertilisation rates with total levels and proportion of sperm exhibiting PLCζ is presented in **Section 4.6**.

4.6 Correlation between total levels of PLCζ and proportions of sperm exhibiting PLCζ with fertilisation rates

In this section, the 30 samples (control and patient) processed during the present study were used to perform a correlation study between PLCζ and fertilisation rates. The 30 samples (control and patient) were supplemented with 16 additional samples from an earlier study carried out by Dr. Junaid Kashir in the Coward laboratory, and then analysed collectively to study the effect of male age upon total levels of PLCζ (**Section 4.7**). This represents the highest number of clinical samples ever analysed for PLCζ.

A comparative analysis to evaluate the link between fertilisation rates and PLCζ were performed. As presented earlier in Section 4.3.2, overall total levels of PLCζ in sperm from patients were reduced by 12% compared to controls (**Figure 4.4**). Similarly, the proportion of sperm exhibiting PLCζ were also reduced by 6% in patients compared to controls (**Figure 4.5**). Although these reductions were not statistically significant ($P \geq 0.05$; as indicated by the Student's *t*-test), the data showed a certain degree of association with reduced rates of fertilisation in controls compared to patients.

Next, fertilisation rates of all 13 controls were compared to the 17 patients. Notably, there was a significant reduction by 53% in fertilisation rates among patients compared to controls ($P \leq 0.05$; as indicated by Chi-Square Test) (**Figure 4.26**). These data collectively showed a clear association between controls and patients in terms of total levels of PLCζ, and the proportion of sperm exhibiting PLCζ, with fertilisation rates. For example, among the controls, the total levels of PLCζ were higher compared to patients (**Figure 4.4**). Correspondingly the mean fertilisation rates were correspondingly higher in controls compared to patients (**Figure 4.5**). Such a clear link between PLCζ and fertilisation rates led us to expand our statistical analysis to evaluate specific correlation between these two variables. Statistical analysis was performed using Pearson correlation and data correlating total levels of PLCζ (**Figure 4.27**) and the proportion of sperm exhibiting PLCζ (**Figure 4.28**) were presented as scatter plot histograms with a linear regression line.

Table 4.15: Fertilisation rate between controls and patients evaluated using Chi-Square test			
Sample	Non fertilised ooytes, (n)	Fertilised oocytes, (n)	Total oocytes, (n)
Controls (n=13)	23	53	76
Patients (n=17)	124	27	151

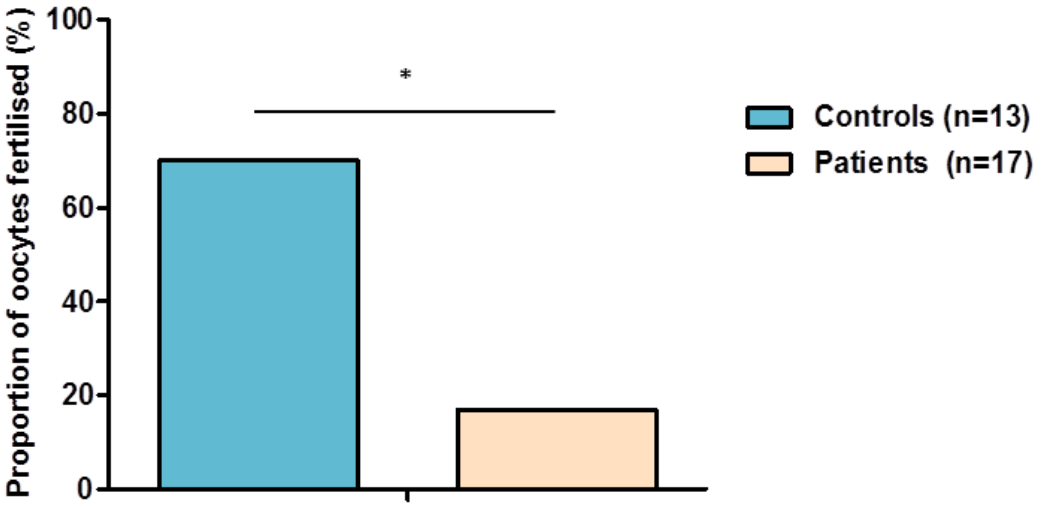


Figure 4.26: Mean fertilisation rates obtained from 13 controls and 17 infertile patients. Fertilisation rates were evaluated based upon the total number of oocyte fertilised following *in vitro* fertilisation and intracytoplasmic sperm injection (ICSI). Asterisk (*) denotes a statistically significant difference ($P \leq 0.05$). Data are shown as percentages.

Table 4.16: Correlation between PLCζ and fertilisation rates				
		Logit transformation_ fertilisation rates (FR)	PLCζ levels	Proportion of sperm exhibiting PLCζ (%)
Logit_FR	Correlation	1.0	-0.8	-0.2
	Significance		0.7	0.4
	N	20.0	20.0	20.0
PLCζ levels	Correlation	-0.8	1.0	0.6
	Significance	0.7		0.0
	N	20.0	30.0	30.0
% PLCζ	Correlation	-0.2	0.6	1.0
	Significance	0.4	0.0	
	N	20.0	30.0	30.0

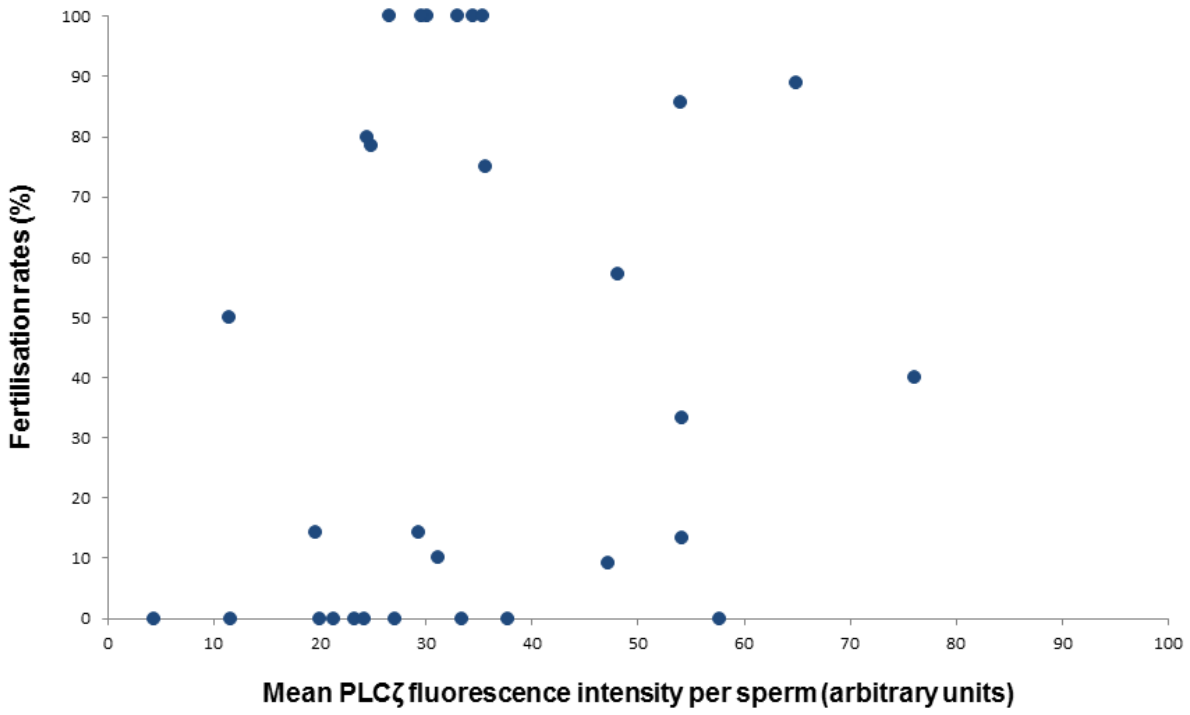


Figure 4.27: Correlation between mean relative total phospholipase C zeta (PLCζ) fluorescence levels and fertilisation rates in sperm from 13 controls and 17 infertile patients. Statistically significant differences ($P \leq 0.05$) were evaluated using Pearson correlation and presented as a scatter plot with a linear regression line. Data are shown as mean total PLCζ fluorescence levels and as percentages for fertilisation rates.

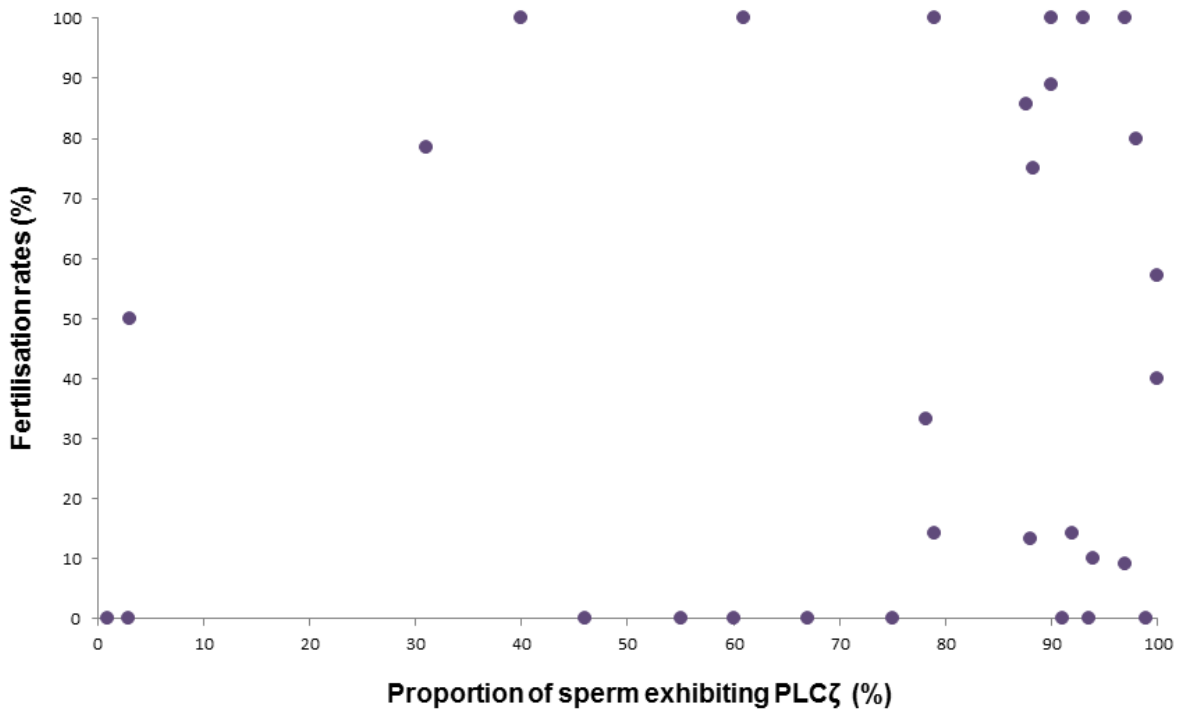


Figure 4.28: Correlation between proportion of sperm exhibiting phospholipase C zeta (PLCζ) and fertilisation rates in sperm from 13 controls and 17 infertile patients. Statistically significant differences ($P \leq 0.05$) were evaluated using Pearson correlation and presented as a scatter plot with a linear regression line. Data are shown as percentages.

The overall conclusion from this dataset is that the total levels of PLCζ (**Figure 4.27**) and the proportional of sperm exhibiting PLCζ (**Figure 4.28**) are both did not show significant correlation with fertilisation rate.

4.7 The effect of male age upon PLCζ levels and the proportion of sperm containing PLCζ in 46 men (23 controls and 23 patients)

Nothing is known of whether male age influences the proportion of sperm exhibiting PLCζ, or the total level of PLCζ in sperm. Here, males were first organised arbitrarily into four age categories (31-35, 36-40, 41-45 and 46 years and above). The total number of controls and patients with their classified age groups, number of sperm analysed and the number of sperm containing PLCζ are presented in **Table 4.17**.

Table 4.17: Total levels of phospholipase C zeta (PLCζ) in four age groups.

Age groups (years)	Number of men (n)	Control (n)	Patient (n)	Total number of sperm analysed	[+PLCζ]	[-PLCζ]	PLCζ levels Arbitrary units (AU)
31-35	12	4	8	1310	841	469	**31.3 ± 3.6**
36-40	20	10	10	2366	1429	937	**37.6 ± 5.4**
41-45	11	7	4	983	812	171	**34.2 ± 3.1**
46 >	3	2	1	305	128	177	**24.1 ± 10.6**
Total	46	23	23	4964	3210	1754	**33.9 ± 2.5**

[+PLCζ] = Total number of sperm exhibiting PLCζ.
[-PLCζ] = Total number of sperm without PLCζ.
Total levels of PLCζ were quantified in arbitrary units (AU).
Data are shown as mean ± SEM.

The total levels and proportion of sperm containing PLCζ were determined in all men by combining the number of sperm from each respective age group. There were no statistically significant differences ($P \geq 0.05$; as indicated by One-Way ANOVA) (**Figure 4.29**) between the age groups in terms of the total levels of PLCζ with a mean of 33.9 ± 2.5AU (**Table 4.17**) or the proportion of sperm exhibiting PLCζ (**Figure 4.30**). Data was then analysed further by sub-dividing samples into two groups (control and patient) and categorising into age groups. The number of controls and patients, with the total number of sperm analysed in each age category are presented in **Table 4.18** and **Table 4.19** respectively.

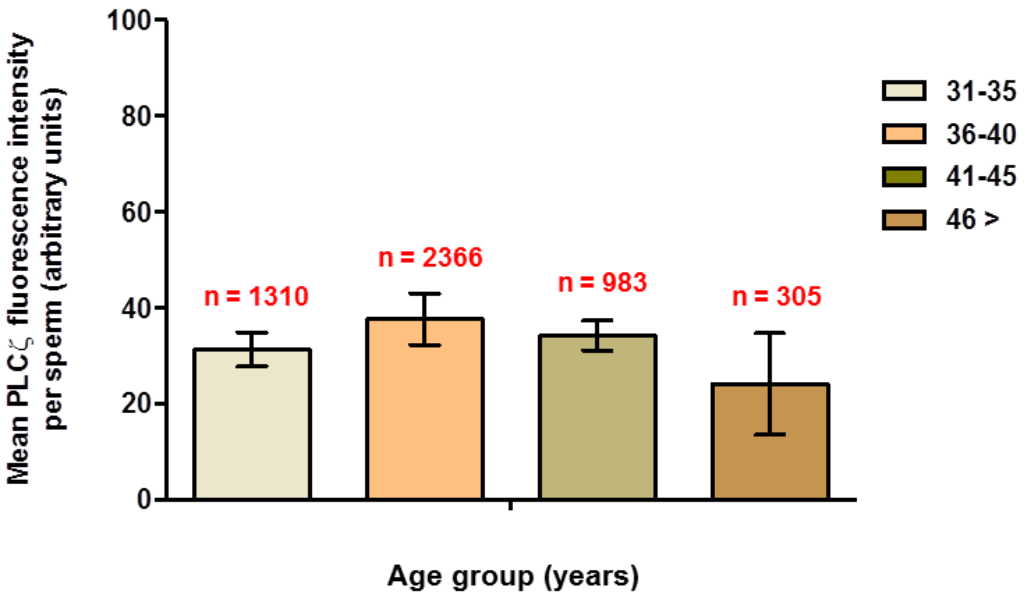


Figure 4.29: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in 46 samples (controls and infertile patients) categorised into age groups. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. There were no statistically significant differences in the total levels of PLCζ between age groups. The numerical values denoted in red font on each histogram represents the total number of sperm analysed. Data are shown as mean ± SEM.

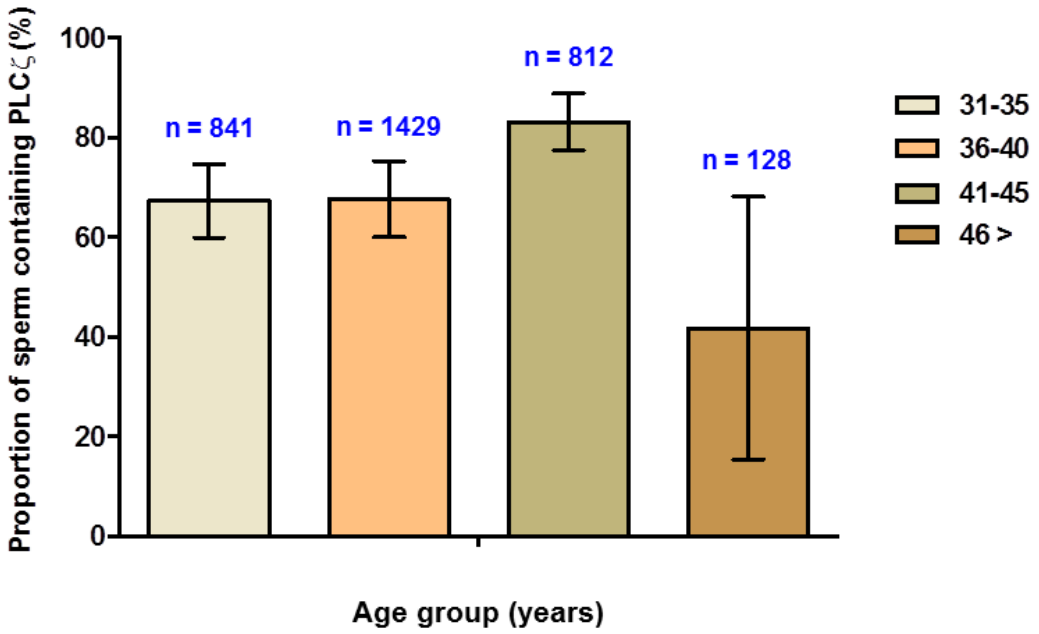


Figure 4.30: Mean proportion of sperm containing phospholipase C zeta (PLCζ) from 46 samples (controls and infertile patients) categorised into age groups. Analysis was performed by ImageJ software. There were no statistically significant differences in the number of sperm exhibiting PLCζ between age groups. The numerical values denoted in blue font on each histogram represents the number of sperm containing PLCζ out of the total number of sperm analysed. Data are shown as mean ± SEM.

Table 4.18: Total levels of phospholipase C zeta (PLCζ) (Mean ± SEM) in controls (n=23) of different age groups.

Age groups (years)	Controls (n=23)	Total number of sperm analysed	[+PLCζ]	[-PLCζ]	Mean PLCζ levels Arbitrary units (AU)
31-35	4	396	264	132	36.1 ± 6.4
36-40	10	1032	833	199	46.7 ± 8.8
41-45	7	633	519	114	33.5 ± 3.2
46>	2	205	127	78	34.0 ± 6.4
Total	**23**	**2266**	**1743**	**523**	**38.9 ± 3.7**

[+PLCζ] = Total number of sperm exhibiting PLCζ.
[-PLCζ] = Total number of sperm without PLCζ.
Total levels of PLCζ were quantified in arbitrary units (AU).
Data are shown as mean ± SEM.

Table 4.19: Total levels of phospholipase C zeta (PLCζ) (Mean ± SEM) in patients (n=23) of different age groups.

Age groups (years)	Patients (n=23)	Total number of sperm analysed	[+PLCζ]	[-PLCζ]	Mean PLCζ levels Arbitrary units (AU)
31-35	8	914	577	337	28.9± 4.3
36-40	10	1334	596	738	31.1 ± 5.4
41-45	4	350	293	57	35.4 ± 7.2
46>	1	100	1	99	4.3
Total	**23**	**2698**	**1467**	**1231**	**29.1 ± 3.2**

[+PLCζ] = Total number of sperm exhibiting PLCζ.
[-PLCζ] = Total number of sperm without PLCζ.
Total levels of PLCζ were quantified in arbitrary units (AU).
Data are shown as mean ± SEM.

Control and patient data from the 46 years and above age group are not shown in the histogram (**Figure 4.31)**. However, there was only one patient (n=1) in this age group, and therefore statistical evaluation was not applicable. Therefore, this group (consisting of 2 controls and 1 patient) were omitted from statistical analysis. However, data for this group is presented separately in **Table 4.18** and **Table 4.19**. As seen with the cumulative analysis (**Figure 4.29**), overall statistical evaluation showed that the total levels of PLCζ in controls and patients with advancing age did not show any significant differences ($P \geq 0.05$; as indicated by One-Way ANOVA) (**Figure 4.31**). However, total levels of PLCζ were significantly reduced among patients compared to controls between 36 and 40 year of age.

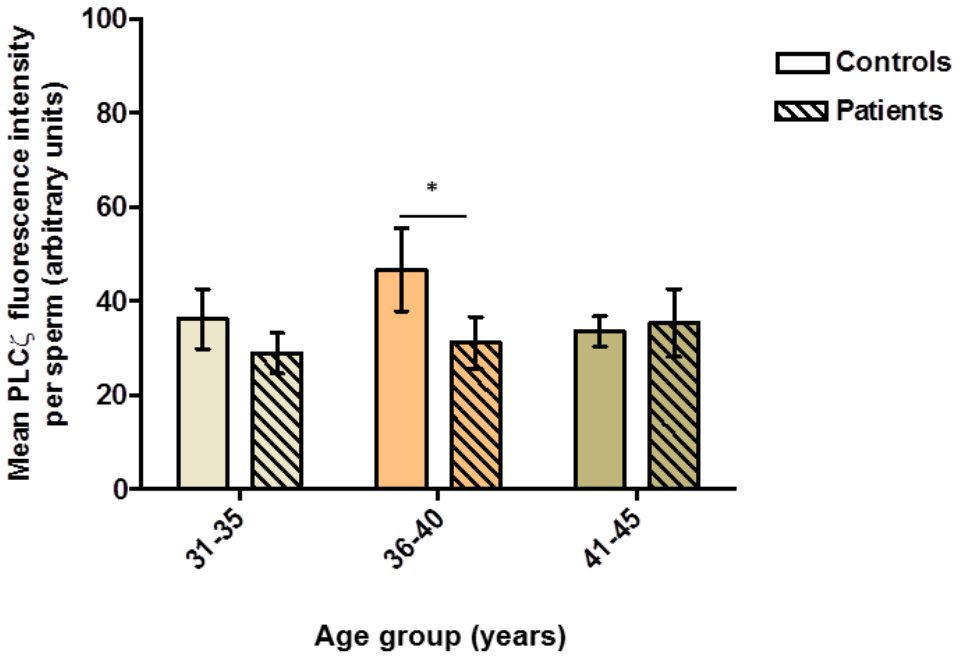


Figure 4.31: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels from 21 controls and 22 infertile patients categorised into age groups. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisk (*****) denotes statistically significant differences ($P \leq 0.05$) between controls and patients in the 36-40 years age group. Data are shown as mean ± SEM.

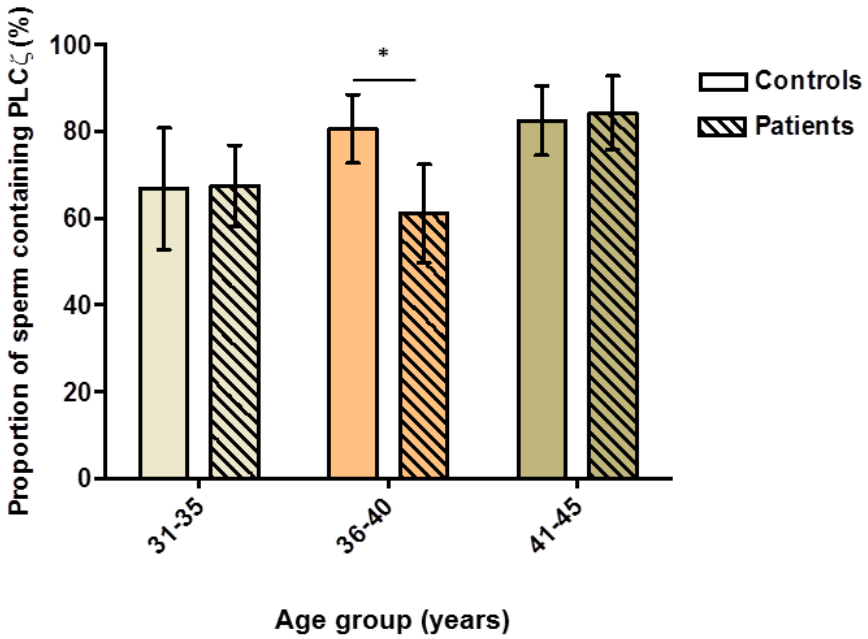


Figure 4.32: Mean proportion of sperm containing phospholipase C zeta (PLCζ) from 21 controls and 22 infertile patients categorised into age groups. Number of sperm exhibiting PLCζ was determined with the use of ImageJ software. Asterisk (*****) denotes statistically significant differences ($P \leq 0.05$) between controls and patients in the 36-40 age group. Data are shown as mean ± SEM.

In the patients there was a significant reduction in the total levels of PLCζ in patients compared to controls in the 36-40 age group. Mean PLCζ levels were reduced by 33% among patients in comparison to controls in this particular age group. Following combination of data from all age groups, the total levels of PLCζ were significantly lower by 25% in patients compared to controls. There were no significant differences ($P \geq 0.05$; as indicated by One-Way ANOVA) identified across the age groups in terms of the proportion of sperm exhibiting PLCζ (**Figure 4.32**).

4.8 Summary of results

1. Total levels of PLCζ and the proportion of sperm exhibiting PLCζ varied significantly between each individual indicating that the presence of PLCζ in a single sperm may fall within a wide range. The localisation patterning of PLCζ showed significant variation both between individuals and within an individual.

2. The total number of sperm analysed in the PLCζ immunofluorescence assay appears to be an essential factor in order to obtain an accurate and consistent statistically significance.
3. Most males exhibited more than one PLCζ localisation pattern as the predominant localisation pattern. The predominant regions for PLCζ localisation were in the P+E or in A+ E+ P regions.
4. Surgical procedures such as vasectomy or vasectomy reversal may cause PLCζ deficiency and sperm structural anomalies leading to OAD or TFF.
5. ICSI failure and OAD may not be completely attributed to PLCζ deficiency, but may also include oocyte-borne factors that interact or regulate the activity of PLCζ.
6. A globozoospermic patient exhibited significant deficiency in PLCζ with uncommon localisation of PLCζ in the mid-piece and anterior region of the sperm head.
7. An SNP was identified in the bi-directional promoter shared by PLCζ and CAPZA3 in one globozoospermic infertile male, and in a non-globozoospermic infertile male. It is possible that this SNP may cause abnormal expression of PLCζ and CAPZA3 resulting in PLCζ deficiency and defects in sperm structure.
8. The development of a PLCζ scoring system based upon defined ranges of data clusters formulated from the entire dataset may represent a valuable biomarker for clinics to predict oocyte activation ability and the likelihood of fertilisation.
9. Overall total levels PLCζ and the proportions of sperm containing PLCζ were significantly reduced in patients compared to controls.
10. Patients with significantly reduced levels of PLCζ exhibited correspondingly low fertilisation rates, compared to controls.
11. Advancing male age does not appear to exert significant effect upon the total levels and proportion of sperm exhibiting PLCζ.

4.9 Discussion

In this chapter, I performed PLCζ screening in a large number of males and attained several fundamental outcomes. PLCζ screening in men seeking infertility treatment may become a vital routine clinical procedure, in order to identify whether poor fertilisation rate or fertilisation failure can be attributed to PLCζ deficiency or any potential genetic anomalies in the PLCζ gene. Herein, it was discovered that total levels of PLCζ and the proportion of sperm containing PLCζ was expressed and localised in a wide undefined range, as reported by Kashir *et al*. (2013b). Interestingly, we defined four ranges of PLCζ which were classified into clusters following statistical analysis. These clusters were used to develop a novel PLCζ scoring system in order to compare the clinical outcome of each control and patient specifically with their PLCζ scores. Next, four special cases were identified which further support the strong link between PLCζ deficiency, genetic abnormalities and poor fertilisation outcomes following ART treatment. Moreover, one of the case studies suggests that surgical procedures in men could also detrimentally affect sperm quality and PLCζ levels leading to fertilisation failure.

While very little is known about the effect of male age upon general infertility treatment outcomes, the present study is the first to investigate whether increasing age could affect the expression levels of PLCζ in the sperm of males across different age ranges. Finally, compilation of the large dataset obtained in this study was used to investigate for specific association between PLCζ and fertilisation rates for the first time. Subsequently, correlation analysis was carried out between the total levels of PLCζ, and the proportion of sperm exhibiting PLCζ, with fertilisation rates in order to develop a correlation tool as a diagnostic or prognostic marker for clinical use.

4.9.1 Significant variability in the total levels and proportion of sperm exhibiting PLCζ in fertile controls and infertile patients

The total levels of PLCζ analysed in sperm from sixteen fertile controls and seventeen infertile patients showed wide variation, from 11.0AU - 54.1AU and from 4.3AU - 64.8AU among controls and patients respectively. These findings support earlier observations reported by Grasa *et al*. (2008) and Kashir *et al*. (2013b). Interestingly, in some cases, the levels of PLCζ were very similar between controls and patients, an observation also reported by Kashir *et al*. (2013b). Proportional analysis also indicated significant variation in the total number of sperm expressing PLCζ within a sample ranging from 13% to 98% and from 1% to 99%, in controls and patients respectively.

Previous studies indicated that different concentrations of PLCζ cRNA were able to elicit Ca^{2+} oscillations in mouse and human oocytes, as seen during normal fertilisation (Cox *et al.*, 2002; Saunders *et al.*, 2002; Rogers *et al.*, 2004; Yoon *et al.*, 2012). Experimental evidence also indicates that the amount of PLCζ present in a single mouse sperm is in the range of 20-50fg (Saunders *et al.*, 2002) and 40-50fg (Fujimoto *et al.*, 2004). Moreover, the amount of mouse PLCζ protein translated from PLCζ cRNA quantified within a mouse oocyte following the microinjection of mouse PLCζ cRNA was approximately 40-80fg (Saunders *et al.*, 2002). Cox *et al*. (2002), further demonstrated that in the mouse oocyte at least, just 4-8fg of human PLCζ was sufficient to elicit Ca^{2+} oscillations leading to oocyte activation and subsequent embryonic development. Therefore, experimental data showing variation in PLCζ protein levels in sperm strongly suggests that there may not be a specific amount of PLCζ presence in a single sperm.

At present, a heterologous ICSI procedure known as the MOAT is used in some clinics to evaluate the oocyte activation ability of a sperm (Vanden Meerschaut *et al*., 2014). Sperm from a specific male is injected into mouse oocytes and the number of oocyte fertilised is evaluated. Subsequently, MOAT groups are classified according to the percentage of oocytes fertilised: 0 to 20% - low, 21% to 84% -intermediate, 85% and above - high (Vanden Meerschaut *et al*., 2014). A recent study demonstrated that sperm from patients, which had been categorised into the three different MOAT categories, elicited different Ca^{2+} oscillatory patterns (Vanden Meerschaut *et al.*, 2013) when injected into mouse oocytes. The distinctive Ca^{2+} oscillatory characteristics of sperm from these different groups of men may therefore be related to the variable amount of PLCζ in such sperm (Cox *et al.*, 2002; Saunders *et al.*, 2002; Kashir *et al.*, 2013b). This is important because differential Ca^{2+} release influences gene expression in developing embryos and can lead to abnormality in the embryo, thus compromising embryo health (Ozil *et al*., 2006). Collectively these data suggest that the amount of PLCζ present in sperm varies markedly among individuals, and in sperm from the same individual. Thus, these findings suggest that the amount of PLCζ required to induce oocyte activation may fall within a wide and as yet undefined range (Kashir *et al.*, 2013b).

Considering the total number of sperm analysed from all controls (n=1243) and patients (n=1972), the total mean fluorescence level, and the proportion of sperm exhibiting PLCζ, were shown to be reduced by 12% and 6% respectively in patients compared to controls; however, these reductions were not statistically significant. These results do not match those of Kashir *et al*. (Kashir *et al.*, 2013b), who demonstrated a significant reduction in the total levels and proportion of sperm containing PLCζ in all infertile patients when compared to fertile controls. However, the difference between the total numbers of sperm and samples analysed by Kashir *et al*. (2013b) in comparison to our study could explain these

contradictory outcomes. For example, in the present experiment, the total number of sperm analysed in controls and patients was 1243 and 1972 respectively, much higher than in Kashir *et al*. (2013b) and there were no statistically significant differences obtained in the total levels of PLCζ or the proportion of sperm containing PLCζ. Moreover, the number of controls (n=13) and patients (n=17) investigated in the present study was much larger compared to the study by Kashir *et al*. (2013b) who analysed 16 controls and only 5 patients. The total number of sperm analysed by Kashir *et al*. (2013b) in patients (n=810) were approximately 50% less than the total number of sperm from controls (n=1690). Moreover, the total levels of PLCζ and proportion of sperm containing PLCζ in patients were also reduced by 50% compared to controls (Kashir *et al.*, 2013b).

Therefore, the previous findings of Kashir *et al*. (2013b), and the results of the present study, strongly indicate that the total number of sperm included in the PLCζ immunofluorescence analysis could be an essential parameter for PLCζ evaluation in order to obtain accuracy and consistency as a prognostic or diagnostic assay. Moreover, it was interesting that most infertile patients analysed here did not exhibit significant deficiency in the total levels and proportion of sperm exhibiting PLCζ. This may indicate that fertilisation failure may not be simply attributable to PLCζ alone, but could also be due to other, as yet unknown factors or idiopathic oocyte problems. The significant variability of PLCζ observed in the large number of males studied herein suggests that there could be specific, and yet unknown mechanisms controlling these wide ranges of PLCζ. While the underlying mechanisms underlying to such variability of PLCζ among the large cohort of males remains unidentified, several possibilities could be put forward. Firstly, the heterogeneous nature of sperm may underlie at least some variability in the expression of PLCζ. This may also explain the significant variability in the localisation of PLCζ seen between individuals and/or within the same individual. Secondly, as previously reported by Saunders e*t al*. (2002) and

Cox *et al*. (2002), the estimated amount of PLCζ protein in an individual sperm varies between 20fg-50fg (Cox *et al.*, 2002; Saunders *et al.*, 2002) and could thus explain the variation of PLCζ observed in the immunofluorescence data described herein (Kashir *et al.*, 2013b). Finally, the polyclonal nature of the PLCζ antibody used herein could also contribute to the observed variability. Specificity of this antibody has been proven extensively. However, production of a PLCζ monoclonal antibody (which is likely to be very specific) would further confirm previous and current findings. While variability in PLCζ is clear and the precise mechanism controlling such variability is very much under debate, it will be fascinating to investigate the Ca^{2+} oscillation profile of sperm exhibiting different levels and localisation patterning of PLCζ following injection of such sperm into mouse oocytes. Such investigations would inform us as to whether any variation in Ca^{2+} releasing ability in response to sperm containing different amount or localisation pattern of PLCζ.

4.9.2 Variation in the localisation patterning of PLCζ in sperm from fertile controls and infertile patients

The localisation of PLCζ in sperm varies across different mammalian species and the presence of PLCζ in distinct regions perhaps indicates multiple functional roles (Fujimoto *et al.*, 2004; Grasa *et al.*, 2008; Young *et al.*, 2009). For example, PLCζ is localised in the acrosomal and post-acrosomal regions of mouse and hamster sperm (Fujimoto *et al.*, 2004; Young *et al.*, 2009). Pig sperm exhibits PLCζ localisation in the post-acrosomal and tail regions (Nakai *et al.*, 2011), whereas PLCζ has been identified in various regions of equine sperm, including the acrosome, equatorial segment, mid-piece and the principal piece (Bedford-Guaus *et al.*, 2011).

The significance of each different localisation pattern of PLCζ in terms of oocyte activation still remains unclear (Kashir *et al.*, 2014). However, in order for PLCζ to carry out

its fundamental role to activate an oocyte, it is crucial that PLCζ is predominantly localised at a readily accessible region within the sperm head which permits rapid dispersion into the oocyte following gamete fusion in order to trigger intracellular Ca^{2+} release and initiate oocyte activation (Nomikos *et al.*, 2012). In line with such a theory, the most suitable region is the equatorial and post-acrosomal region since these are the first to come into contact with the oocyte following gamete fusion (Parrington *et al.*, 1996; Kashir *et al.*, 2014). Several studies have reported data in human sperm that support the predominant localisation of PLCζ in these regions (Grasa *et al.*, 2008; Kashir *et al.*, 2013b). This is also true of the data presented in this thesis. In the human sperm analysed during the current study, PLCζ was localised in three distinct regions of the sperm head: the acrosomal, equatorial and post-acrosomal regions. Furthermore, it was evident that these localisation patterns exist in a variety of combinations without uniform distribution. Previous studies also reported that the specific localisation of PLCζ in human sperm shows variation among different individuals but was predominantly localised in the equatorial (E) and post-acrosomal + equatorial (P+E) regions within the sperm head (Grasa *et al.*, 2008; Kashir *et al.*, 2013b). In the present study using a significantly larger number of males, it was evident that the predominant localisation pattern in sperm from controls and patients was a combination of P+E and A+E+P. Consistent with Kashir *et al*. (2013b), the present study observed seven different patterns of PLCζ localisation, including a variety of combinations, among the three principal areas of localisation (acrosomal, post-acrosomal and equatorial) regions. Sperm from the majority of controls and patients exhibited a single predominant localisation pattern of PLCζ, and some individuals whether (control or patient) demonstrated more than one pattern as the predominant localisation pattern, which corresponds to the previous findings of Kashir *et al*. (2013b).

The significance of the equatorial and post-acrosomal region of PLCζ in human sperm for oocyte activation is clearly evident as these are the regions that meet the oocyte first. However, the fact that PLCζ is found in the acrosomal region, and in different combinations, may suggest differential functional roles apart from oocyte activation, such as capacitation and the acrosome reaction (Fujimoto *et al.*, 2004; Grasa *et al.*, 2008; Young *et al.*, 2009). Another example is that the acrosomal population of NYD-SP27 (an isoform of PLCζ) in human and mouse sperm has been demonstrated to function as an intrinsic de-capacitation factor which prevents premature capacitation (Bi *et al.*, 2009). The specific mechanism underlying these subtle differences in PLCζ localisation patterning remain unknown, and require urgent investigation to conclusively determine the exact role of such patterning and whether different populations perform different functions. Such investigations need to determine if these observations are attributed to the natural heterogeneity of sperm or the polyclonal nature of the antibody used in this and earlier studies, although the specificity of the antibody has been extensively proven by PLCζ immunogenic peptide blocking experiments using the same immunofluorescent technique in human sperm (eg. Grasa *et al.* 2008). Since sperm from different men exhibits differential Ca^{2+} oscillation patterning in mouse eggs following heterologous ICSI (Vanden Meerschaut *et al.*, 2013), it would be interesting, if future work investigates whether sperm exhibiting different populations of PLCζ also elicit variations in Ca^{2+} oscillation patterning.

4.10 Case studies

4.10.1 Case study # 1: Patient 6 - The potential effect of vasectomy/vasectomy reversal upon sperm morphology and total levels of PLCζ

The current study is the first to demonstrate significantly reduced levels of PLCζ in sperm from a man experiencing vasectomy reversal. Moreover, morphologically defective sperm were also identified in this particular case. Vasectomy involves the removal of the vas deferens (a tissue that connects the testis and epididymis) (Bedford *et al.*, 1973; Thimon *et al.*, 2007). The epididymis facilitates sperm maturation including the acquisition of progressive motility and fertilisation ability of the sperm (Cooper, 2007; Cornwall, 2009) and acts as a reservoir for sperm. These biochemical modifications, referred to as "sperm maturation" are carefully orchestrated along the segmental epididymis (caput, corpus and cauda) and are highly dependable on appropriately regulated gene expression patterns under the influence of steroid hormones, lumicrine factors and small non-coding RNAs (Cornwall, 2009; Belleannee *et al.*, 2012).

The microenvironment (flux composition and epididymal fluid) within the segments of the epididymis facilitates sperm maturation events. Following vasectomy, the composition of the specialised microenvironment within the epididymis is interrupted, thus affecting the sperm membrane and consequently the fertilising ability of sperm (Belleannee *et al.*, 2013). Disruption of the vas deferens upon vasectomy has been demonstrated to alter gene and protein expression profile within the epididymal fluid, which plays a major role in sperm maturation (Thimon *et al.*, 2008; Sullivan *et al.*, 2011). Failure of the epididymis to rescue such alterations following vasovasostomy, which affects the functional ability of a sperm for

fertilisation, may explain the discrepancy between surgical success and poor pregnancy outcome (Sullivan *et al.*, 2011; Belleannee *et al.*, 2013).

Previous studies using a mouse model indicated that vasectomy leads to significant changes in epididymal morphology and markedly reduces sperm motility, thus leading to poor fertilisation outcome (Mahabadi *et al.*, 2013). This finding further led the authors to suggest the overall effect of vasectomy upon reduced fertilisation rate. Variation in chromatin remodelling in association with alterations in protamine expression were also demonstrated in human males, including vasectomy reversal males (de Vries *et al.*, 2013). While poorly condensed sperm nuclear chromatin and alterations in protamine content were shown to associate with infertility and markedly reduced fertilisation rates (Francis *et al.*, 2014), one particular study, using a rabbit model, demonstrated spermatogenic arrest and poorly condensed chromatin structure in sperm following vasectomy. This study also revealed the delayed expression of P1 and P2 (Steger *et al.*, 2005), which are vital in the preservation of DNA integrity in sperm (Francis *et al.*, 2014).

Sperm nuclear condensation and protamine content is not only vital for the protection of sperm genomic materials, but is also crucial in the formation of the hydrodynamic structure with which to maintain an intact sperm head (Francis *et al.*, 2014). There is also evidence demonstrating that vasectomy/vasectomy reversal could detrimentally affect sperm nuclear condensation and protamine expression, leading to alteration in the hydrodynamic formation of the sperm head (de Vries *et al.*, 2013). This could adversely affect sperm morphology, which may explain the morphological abnormalities in 37% of the sperm observed in the present case study, a 39 year old man seeking ART treatment following vasectomy reversal. PLCζ levels were also significantly reduced in the 37% of sperm showing abnormal morphology. According to several reports, it is evident that sperm with

abnormal morphology routinely show distorted, abolished, punctuated or translocated forms of PLCζ (Kashir *et al.*, 2012c; Lee *et al.*, 2014).

Furthermore, patient 6 in the present study exhibited 63% of sperm with morphologically normal sperm, which also exhibited significantly reduced levels of PLCζ compared to controls. Evidence shows that there are many changes in the biochemical and molecular activity of the testis and epididymis in vasectomized men, and that following reversal, the chances of recovery are low (McVicar *et al.*, 2005). Such changes may increase the level of oxidative stress in the epididymis during sperm maturation, a factor known to cause infertility in men when sperm and seminal fluid are exposed to ROS (Agarwal *et al.*, 2014). Persistent infertility in response to oxidative stress following vasectomy reversal has been demonstrated in several studies (Kolettis *et al.*, 1999; Liu *et al.*, 2014). Collectively, alterations in the biochemical process and increased levels of oxidative stress along the epididymis, may ultimately have affected the expression of PLCζ in patient 6, thus associating for his deficiency.

The precise biochemical and molecular mechanism of vasectomy reversal in relation to infertility remains unclear (Belleannee *et al.*, 2013). Thus far, research has clearly associated fertility defects in vasectomy and vasectomy reversal patients with various factors including changes in the male reproductive tract, obstruction, epididymal gene or protein expression profile, oxidative stress, sperm motility, integrity and protein structure or function. It is also evident that vasectomy reversal often results in infertility, yielding poor pregnancy outcome, even when treated by IVF or ICSI (van Dongen *et al.*, 2012). Although a potential link between vasectomy/vasectomy reversal and total levels of PLCζ has been clearly demonstrated in this chapter, at least in this particular individual, it is critical that future studies investigate whether any fertility-related surgical procedure, including surgical sperm retrieval (SSR), could also affect PLCζ. Such investigations are crucial not only to expand the

current findings of this thesis, but also to establish whether such procedures may distort/abolish the expression of PLCζ leading to oocyte activation and fertilisation failure. Thus far, only one patient undergoing vasectomy/vasectomy reversal has been screened for PLCζ, which limits our conclusion that such surgical procedure could routinely effect the expression of PLCζ. Therefore, it is crucial to increase the sample size of men with such conditions to further prove our current finding. Treatment options such as AOA application, or recombinant PLCζ therapy, can be recommended for this patient in order to restore oocyte activation and fertilisation rate. Heterologous ICSI can be performed using sperm form this patient and co-injection with PLCζ cRNA, or PLCζ pure protein into mouse to evaluate oocyte activation and fertilisation rate. Further genetic screening of PLCζ gene in both the promoter and exonic regions in this patient are crucial as it would enable us to identify whether PLCζ deficiency is attributed to genetic abnormalities, particularly, whether surgical procedure could detrimentally effect the molecular expression of PLCζ.

4.10.2 Case study # 2: Patient 7 - Successful fertilisation following the application of AOA in a PLCζ-deficient patient and identification of a single nucleotide substitution in the PLCζ promoter region

The implementation of ICSI is widely accepted as the most reliable treatment for severe male infertility (Palermo *et al.*, 2009); however ICSI treatment does not necessarily lead to successful fertilisation (Flaherty *et al.*, 1998; Esfandiari *et al.*, 2005). Although there are various factors attributed to ICSI failure, OAD is considered as the most common factor underlying fertilisation failure following ICSI (Vanden Meerschaut *et al.*, 2014). Deficiency in the levels of PLCζ has been demonstrated in patients diagnosed with OAD in previous studies (Yoon *et al*., 2008, Heytens *et al*., 2009, Kashir *et al*., 2013b). In the present study, we investigated patient 7, who encountered TFF following ICSI. Patient 7 was initially

diagnosed with unexplained infertility, however our PLCζ analysis revealed that sperm from this patient exhibited significantly reduced levels of PLCζ compared to a control, which suggested that ICSI failure in this patient was very likely to be attributed to OAD.

It is well established that ICSI failure can be rescued by the application of artificial oocyte activators (AOA) (Heindryckx *et al.*, 2008; Vanden Meerschaut *et al.*, 2012). The type of AOA used to rescue ICSI failure cases include Ca^{2+}- ionophores (Heindryckx *et al.*, 2005; Heindryckx *et al.*, 2008) and strontium chloride (Yanagida *et al.*, 2008). The most commonly used AOA to activate a human oocyte are Ca^{2+}- ionophores such as ethanol, ionomycin, and A23187 frequently used in combination with protein synthesis or kinase inhibitors such as 6-dimethylaminopurine (6- DMAP) or puromycin that blocks the re-synthesis of cyclin B or CDK1 activity (Heindryckx *et al.*, 2007; Ramadan *et al.*, 2012).

As the beneficial aspect of AOA application to improve fertilisation rate of ICSI failure cases is well-known (Vanden Meerschaut *et al.*, 2014), and following our diagnosis of PLCζ deficiency, the patient entered another cycle of ICSI in combination with AOA. Fortunately, the couple attained successful oocyte activation with a 54% fertilisation rate and an embryo was implanted. This finding does not only support existing studies which indicates the successful application of AOA in such cases, but also further demonstrates the link between PLCζ deficiency in sperm and OAD, and that an alternative agent can be used to trigger Ca^{2+} oscillations within the oocyte and thus restore fertility.

However, concerns remain with regard to the application of AOA during ICSI, since these agents do not mimic the physiological Ca^{2+} oscillations seen during normal mammalian fertilisation (Amdani *et al.*, 2013; Kashir *et al.*, 2014). Moreover, the use of strontium chloride triggers the periodic release of Ca^{2+} voiding IP_3 production (Kashir *et al.*, 2014). However, the application of AOA during ICSI has been successful thus far (Kashir *et al.*,

2010), especially in treating severe male infertility cases such as globozoospermia patients whom resulted with high fertilisation and pregnancy rates (Taylor *et al.*, 2010). This may also suggest that there is a clear need to produce a physiological oocyte activation agent, which permits periodic Ca^{2+} release in oocyte without affecting gene expression during embryonic development, such as purified PLCζ protein. A recent study has shown that purified human PLCζ protein is able to restore oocyte activation in mouse oocytes that had previously been microinjected with mutant forms of human PLCζ (Nomikos *et al.*, 2013a).

Numerous reports describe a clear link between PLCζ deficiency and male infertility, especially in terms of ICSI failure and OAD (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Kashir *et al.*, 2013b). Additionally, an OAD patient exhibiting two mutations in the exonic regions of the PLCζ gene was also identified in previous studies (Heytens *et al.*, 2009; Kashir *et al.*, 2012b). Recently, mutations in the PLCζ gene promoter region in Chinese Holstein bull, which altered semen quality traits such as semen ejaculated volume, sperm motility and deformity, were reported (Pan *et al.*, 2013). All previous studies have focused upon screening exonic regions of the PLCζ gene for mutations. However, the findings of Pan *et al.* (2013) led to our laboratory performing new genetic screening protocols to look for potential anomalies in the PLCζ promoter region in human sperm. Importantly, patient 7 exhibited a SNP in the PLCζ promoter region. This represents the first PLCζ mutation discovered out of the coding region and only the second patient ever to be identified as having a PLCζ genetic anomaly. However, it is essential to expand this finding in a larger group of men, to assess whether such SNPs can influence oocyte activation ability leading to fertilisation failure. Moreover, these investigations may allow further understanding of the functional role and genetic regulation of PLCζ in both, fertile and infertile men. It is crucial for future studies to perform genetic screening in the promoter, intronic and exonic regions of PLCζ gene to identify if there are any genetic anomalies such as mutations or SNPs, and if such anomalies are

generally found in infertile men with PLCζ deficiency or even in OAD and ICSI failure patients. SNPs identified in the PLCζ promoter region, which is responsible for the regulation of transcription, may alter the gene expression profile, consequently affecting PLCζ structure/function, and may even be responsible for the variability of PLCζ seen in the large number of men studied in the present project.

4.10.3 Case study #3: Patients 8 and 9 – Could the dysfunction of a potential unknown oocyte factor underlie ICSI failure in patients exhibiting OAD, but normal levels of PLCζ?

While the clear link between OAD, ICSI failure and PLCζ deficiency in infertile males has been widely reported (Amdani *et al.*, 2013), it was interesting to encounter ICSI failure patients in the present study who did not appear to exhibit PLCζ deficiency. In the current study, both patient 8 and 9 demonstrated TFF or low fertilisation rate following ICSI, although the total levels of PLCζ and the proportion of sperm exhibiting PLCζ were comparable with a control. Considering the overall evidence reported, there is a strong likelihood that an as yet unidentified oocyte factor within the ooplasm may play a fundamental role in activating PLCζ to hydrolyse vesicular bound PIP_2 to induce Ca^{2+} oscillations (Nomikos *et al.*, 2013b). This may imply that patients 8 and 9 had abnormalities in this oocyte-borne factor, although a donor oocyte also resulted in low fertilisation rates, suggesting this might not necessarily be the case with one of the patient.

A recent immunofluorescent study demonstrated PLCζ localisation in small intracellular vesicles within the ooplasm suggesting PLCζ induced Ca^{2+} oscillations via an IP_3 signalling pathway initiated by interaction with an unknown receptor on the vesicles (Yu *et al.*, 2012). One potential suggestion might be that dysfunction of this unknown oocyte factor may inhibit the action of PLCζ by maintaining its inactive state leading to failure of the

oocyte activation mechanism, even though PLCζ may have successfully diffused into the oocyte. It is possible that patient 8 did not exhibit oocyte factor deficiency, since there was an 18% fertilisation rate obtained (which is still considered low). Patient 9 is more likely to exhibit oocyte-borne factor deficiency since the sperm from patient 9 were able to successfully fertilise donor oocytes. However, the present results could not firmly conclude deficiency of an unknown oocyte factor. It is possible that our PLCζ immunofluorescence assay may also have limited power in detecting OAD in these patients. Although, our immunofluorescence assay showed comparable levels of PLCζ to controls, the functional ability of this sperm is not known and would require Ca^{2+} releasing ability experiments to be carried out. The Coward Laboratory is currently trying to establish a calcium release assay in house to help elucidate functional ability in such patients.

4.10.4 Case study # 4: Patient PM - Deficiency and novel localisation of PLCζ in a globozoospermic patient and a possible link to an SNP in the PLCζ promoter

Male factor infertility is generally attributed to abnormal seminal/sperm parameters such as sperm motility, concentration and morphology. Such conditions are categorized as azoospermia (low sperm count), oligozoospermia (poor sperm concentration), asthenozoospermia (poor sperm motility) and teratozoospermia (abnormal sperm morphology). Frequently, idiopathic reasons are suggested to be the principal factor of male infertility (Kashir *et al.*, 2010). Another common morphological defect identified in infertile men is globozoospermia, a condition where sperm exhibit a round head and lack an acrosome. This condition affects 0.1% of infertile men (Dam *et al.*, 2007a) and is a devastating condition. Such sperm are not only considered to be morphologically deformed, but also generate aberrant Ca^{2+} oscillations and are routinely oocyte activation deficient

(Heytens *et al.*, 2009; Kashir *et al.*, 2010). Several studies have demonstrated links between PLCζ deficiency and globozoospermia (Heindryckx *et al.*, 2005; Heytens *et al.*, 2009; Taylor *et al.*, 2010; Kashir *et al.*, 2012c). While the precise mechanism linking globozoospermia and PLCζ deficiency is poorly understood, such sperm encounter significant distortion within the head, perinuclear theca and the equatorial region, leading to translocation of PLCζ, subsequently affecting the ability to induce Ca^{2+} oscillations (Lee *et al.*, 2014). Indeed, in many cases, globozoospermia men are completely devoid of PLCζ.

Earlier studies have shown deficiency or complete absence of PLCζ in sperm from globozoospermic patients, which exert abolished and abnormal forms of Ca^{2+} release when injected into mouse oocytes (Yoon *et al.*, 2008, Heytens *et al.*, 2009). While ICSI is able to treat many forms of male infertility (Vanden Meerschaut *et al.*, 2014), unfortunately the fertilisation rate following ICSI is poor in severe male conditions (Vanden Meerschaut *et al.*, 2012), especially with globozoospermia and patients with genetic abnormalities (Amdani *et al.*, 2013). The use of advanced techniques such as MSOME enables the selection of sperm from globozoospermic patients exhibiting an acrosomal bud, and the subsequent application of IMSI has led to successful fertilisation and clinical pregnancy (Sermondade *et al.*, 2011). Further investigation using immunofluorescence techniques revealed that MSOME-selected sperm exhibiting acrosomal buds showed novel localisation patterns of PLCζ in the acrosomal region and mid-piece (Kashir *et al.*, 2012c). From this finding, it was evident that PLCζ may serve as a clinical therapeutic tool and that the application of MSOME/IMSI in globozoospermic conditions may not necessarily require AOA treatment.

In the present study, patient PM, a 43 year old man was recruited from Belgium and underwent three ICSI cycles in combination with AOA treatment. The couple had a successful singleton delivery following the 3rd ICSI cycle. Total levels of PLCζ and the proportion of sperm exhibiting PLCζ in the patient was significantly reduced compared to a

control. PLCζ was localised at the anterior and mid-piece region of the globozoospermic sperm from patient PM. These findings concur with those reported by Kashir *et al*. (2012c) who demonstrated a novel localisation of PLCζ in sperm from several globozoospermic patients. Localisation of PLCζ in the mid-piece is very unusual and may be related to abnormalities in sperm structure or the molecular pathway involved in expression of PLCζ. Moreover, globozoospermia is a complicated genetic condition, since mutations in two other genes such as spermatogenesis-associated protein 16 (SPATA16) and DPY-19-like 2 (DPY19L2) has been frequently identified in globozoospermic patients (Dam *et al.*, 2007b; Koscinski *et al.*, 2011). Both SPATA16 and DPY19L2 are highly expressed in testis and play a major role during spermatogenesis, especially DPY19L2 which is predominantly involved in sperm head elongation and acrosome formation. Interestingly, PLCζ shares a bi-directional promoter with CAPZA3 gene, which is predominantly involved in structural formation of the developing spermatid (Geyer *et al.*, 2009; Coward *et al.*, 2011). Evidently, studies have demonstrated that mutation of CAPZA3 causes disruption of the F-actin network and disorganisation of sperm structure of mouse sperm leading to an aberrant globozoospermia prototype. Moreover, mice exhibiting this mutation were infertile (Geyer *et al.*, 2009). It is possible, therefore the deficiency in PLCζ observed in patient PM in the current study may also have abnormalities in CAPZA3 due to a potential defect in the bi-directional promoter region, which may explain the resulting translocation of PLCζ to the mid-piece or anterior region of the sperm.

Bi-directional promoters are predominantly involved in regulating the co-expression of genes that function in a similar biological pathway, or coordinate responses to induction signals (Trinklein *et al.*, 2004). However, the specific role of the bi-directional promoter region shared by PLCζ and CAPZA3 in terms of gene expression and potential functional coordination role is largely unknown. It was interesting that another crucial result obtained

from this patient PM, who is a globozoospermic, is the identification of an SNP in the promoter of PLCζ gene as seen in patient 7, who do not exhibit globozoospermia (**Case study #2**). The SNP identified in the PLCζ promoter region may suggest that this does not only effect PLCζ levels in sperm, but could also exert morphologically abnormalities leading to globozoospermia via the inappropriate expression of CPAZA3. There are possibilities where SNPs in the promoter region can generally affect the overall PLCζ and CAPZA3 gene transcriptional and translational activity, consequently compromising the structure and function of both proteins. However, this hypothesis remains untested, until several rudimentary experiments are performed. For example, the immunofluorescence assay can be performed simultaneously to evaluate both PLCζ and CAPZA3 in sperm from fertile or infertile men in order to determine the expression levels of both these proteins. Next, molecular cloning technology can be applied using a dual reporter construct plasmid to evaluate the expression of both PLCζ and CAPZA3 proteins in a mammalian cell line such as HEK cells. Such experiments would first enhance our understanding of the expression pattern of both proteins prior to investigating their functional role and the underlying mechanism in relation to male infertility.

Currently, there have been 1338 SNPs identified in the PLCζ gene (data obtained from the short genetic variation database, National Centre for Biotechnology Information) but the effect of these SNPs upon male factor infertility is not known. It will be interesting for future studies to recognise and collect data from all of these SNPs and perform genetic screening in patients with OAD, ICSI failure and globozoospermia condition, or even in fertile controls. This would a provide a large dataset to investigate whether SNPs could exert detrimental effects upon the oocyte activation ability of sperm, expression of PLCζ, morphologically abnormalities, or whether SNPs can actually improve the function of PLCζ. Notably, it is possible that the variability in total levels of PLCζ, proportional analysis, and

localisation patterning of PLCζ could be attributed to the presence of SNPs in the PLCζ promoter.

4.11 Development of a novel PLCζ scoring system to predict oocyte activation deficiency infertility and to compare fertilisation outcome following ART

The fundamental role of PLCζ upon determining male infertility, especially in terms of oocyte activation ability of sperm is clearly evident. The total level of PLCζ exhibits significant variability in human sperm, and in some cases, similar levels of PLCζ were identified in controls and OAD patients as demonstrated herein and by Kashir e*t al*. (2013b). From the collective results obtained from 30 males in this chapter and following cluster analysis (**Section 4.3.2**), a potential PLCζ scoring system was derived and constructed as a prototype assay for clinics. Approximately 43% of males (controls and patients) achieved a PLCζ score exhibiting low levels of PLCζ with higher proportion of sperm exhibiting PLCζ (#). Fertilisation rates following IVF or ICSI from these men varied significantly and did not directly correspond to the levels of PLCζ when compared individually.

For example, three patients scored low levels of PLCζ with higher proportion of sperm exhibiting PLCζ (#). PLCζ levels demonstrated TFF and 3 other patients with a similar score showed very low fertilisation rates which fall in a range of 10% to 14%. Since the clinical diagnoses for most of these patients remain unexplained, the current findings suggest that fertilisation failure following IVF/ICSI may not necessarily be attributed solely to the level of PLCζ, but are likely to include a variety of other factors such as sperm de-condensation defects or potential unknown oocyte problems linked to the IP_3 molecular pathway, sperm-oocyte interaction deficiency or the interaction of PLCζ with unidentified oocyte-borne factors within the ooplasm.

From this study, two patients showed TFF and exhibited the lowest PLCζ score (*), while 46% of the controls scored intermediate (**) to high (***) PLCζ scores with high fertilisation rates, thus suggesting a clear link between fertilisation rate and levels of PLCζ at least in some cases. Utilisation of the proposed PLCζ scoring system to predict oocyte activation ability and fertilisation outcome remains highly useful but with the caveat that there is a high degree of variation in total levels of PLCζ and the number of oocytes fertilised when the present data were compared individually between each male. However, when the levels of PLCζ were compared collectively with fertilisation rates, a strong positive significant correlation was obtained, indicating that PLCζ levels may serve as a potential biomarker to predict fertilisation rate. Correlative studies between the total levels of PLCζ, proportion of sperm containing PLCζ, and fertilisation rates are further discussed in the following section.

4.12 Correlation between total levels of PLCζ and proportions of sperm exhibiting PLCζ with fertilisation rates

Compilation of data from a group of 30 men studied herein firstly showed that total levels of PLCζ and the proportion of sperm exhibiting PLCζ in patients were significantly reduced compared to controls. This finding is similar to that reported by Kashir *et al*. (2013b). However, Kashir *et al*. (2013b) did not perform a correlation or association study between PLCζ and fertilisation rates (Kashir *et al.*, 2013b). In the present study, there was a clear association observed between the fertilisation rates of controls and patients with their corresponding mean PLCζ levels and PLCζ proportional analysis. Therefore, correlation analysis was carried out and revealed strong correlations between fertilisation rates following IVF/ICSI and total levels of PLCζ and the proportion of sperm containing PLCζ. While, Lee *et al*. (2014) recently demonstrated a clear link between reduced PLCζ levels and fertilisation

failure following ICSI (Lee *et al.*, 2014), to our knowledge, the present study is the first to show PLCζ correlation analyses with fertilisation rate following both IVF and ICSI treatment. Current data did not show correlation between PLCζ and fertilisation rate indicating that fertilisation following IVF may be attributed to other factors such as acrosome reaction, sperm penetration and sperm decondensation apart from PLCζ alone.

4.13 The effect of male age upon PLCζ levels and the proportion of sperm containing PLCζ in 46 men (23 controls and 23 patients)

Advancing maternal age is frequently associated with a higher risk of infertility, which predominantly involves a gradual reduction in ovarian reserve and oocyte integrity (Belloc *et al.*, 2014) leading to miscarriage and chromosomal abnormalities (Humm & Sakkas, 2013). In addition, women may also encounter pre-natal and post-natal complications with advancing age (Jacobsson *et al.*, 2004). Collectively, these events may explain the reduced reproductive capacity and poor ART outcome in aged women (Pal & Santoro, 2003).

While the higher risk of infertility with increasing maternal age is widely accepted, the decline in reproductive capacity in males with advancing age is also undeniable, but is less well characterized. Reports have shown clear associations between increasing paternal age with higher infertility risk such as alteration in reproductive hormonal secretion, sperm production and function (Belloc *et al.*, 2014). For example, changes in sperm parameters such as reduced sperm count and motility, or increased prevalence of morphological abnormalities have also been demonstrated (Kidd *et al.*, 2001; Eskenazi *et al.*, 2003; Sloter *et al.*, 2006). Although semen analysis is a well-established clinical diagnostic tool to predict male infertility, there is high biological variation in terms of actual clinical outcomes (Guzick *et al.*, 2001; Keel, 2006). This represents one of the disadvantage of basic semen analysis as a predictor for male infertility (Belloc *et al.*, 2014). Semen analysis only involves assessment

of motile sperm counts, movements or morphological abnormalities and is not able to evaluate the molecular integrity of sperm, such as sperm DNA integrity, viability or even oocyte activation ability. Therefore, it is highly prudent to develop and utilise new biological tools to specifically analyse sperm proteins, for example PLCζ (Kashir *et al.*, 2013b) or the extent of sperm DNA fragmentation, as supplementary tests to the conventional semen analysis (Simon *et al.*, 2010; Zini, 2011; Humm & Sakkas, 2013).

Numerous studies have demonstrated strong links between male infertility and increasing levels of sperm DNA fragmentation (Spano *et al.*, 2000; Giwercman *et al.*, 2010). Men with abnormal sperm parameters have been shown to have significantly increased levels of DNA fragmentation which resulted in poor or failed fertilisation outcome and compromised embryonic development following infertility treatment (Sakkas & Alvarez, 2010; Humm & Sakkas, 2013). Interestingly, higher levels of DNA fragmentation have been associated with increasing paternal age by several studies (Hammiche *et al.*, 2011; Varshini *et al.*, 2012; Das *et al.*, 2013). While most studies have shown an age-related effect upon DNA damage in infertile men, data has also highlighted that healthy males of advanced age also demonstrate increased levels of DNA fragmentation as compared to younger men despite having normal semen parameters (Wyrobek *et al.*, 2006). While the adverse effect of male age upon DNA integrity is clearly evident, it remains unknown whether age factors may affect the integrity, expression or function of crucial sperm proteins such as PLCζ or protamines. For example, several studies have shown poor DNA integrity in sperm from aged fertile and infertile males. Since sperm DNA integrity is established by protamines (Francis *et al.*, 2014), it is worrying that there have been no studies as yet that have investigated possible links between male age and protamine expression and function in human sperm. While DNA fragmentation testing may serve as a useful biological tool for male infertility assessment

(Simon *et al.*, 2010), the evaluation of sperm protamines is likely to represent a valuable addition to the clinic as a diagnostic or prognostic indicator (Nanassy *et al.*, 2011).

To our knowledge, this is the first study to specifically correlate total levels of PLCζ with male age in a large population. Following comparative statistical analysis across different age groups, the resultant data failed to demonstrate any significant differences in the total levels of PLCζ or the proportion of sperm exhibiting PLCζ in a given sample, although there was a general trend for reduced total levels of PLCζ with age. These findings strongly suggest that there is no statistical effect of male age upon PLCζ levels. However, there was significant variation in total levels of PLCζ between each individual within their respective age grouping and it is not known whether this influenced the outcome of the analysis. Inconsistency and variation in the number of males analysed, including the total number of sperm analysed within each age group, may limit the accuracy of the results obtained. For example, for the 36-40 age group, 2366 sperm were analysed compared to only a total of only 305 sperm from the 3 males analysed in the 46 years and above age group. Nevertheless, this is an interesting and original finding and suggests that the level of PLCζ does not change significantly with age.

A general trend for reduction in the total levels of PLCζ was observed starting from the age of 36-40 years to 41-45 years in controls, although this was not statistically significant. Furthermore, the total levels of PLCζ were significantly reduced in patients compared to controls within the 36-40 year age group. Similar significance was obtained for the 46 years and above age group, although there was only one patient in this age group. In addition, the eldest patient examined here exhibited significantly lower levels of PLCζ than all other patients. Considering the proportional analysis, there was a significant difference observed in the number of sperm containing PLCζ between controls and patients only in 36-40 year age group.

As described earlier, the inherent variability observed within the present population, as also reported by Kashir *et al*., (2013b), and discussed in **Section 4.3.2**, is an important factor to consider. Therefore, it is prudent for future studies to investigate the effect of male age upon PLCζ in a larger number of men within each age group to obtain a more representative picture and increase statistical power. Such investigations may not only enhance our understanding of PLCζ with advancing male age, but may also be highly beneficial for ART clinics as an indicator for potential oocyte activation ability. Although in the present study there was no clear association obtained between the total levels of PLCζ and proportion of sperm containing PLCζ in relation to increasing male age, it is important to remember that Ca^{2+} release ability across age groups was not tested. Moreover, sample size was too small to indicate trends between genetic abnormality and age. It will be interesting to incorporate these experimental approaches to acquire an in depth understanding of the functional role of PLCζ in men of different ages. This may also dictate the degree of oocyte activation ability of sperm from different ages.

4.14 Summary and future directions

The field of ART continues to grow with the implementation of sophisticated techniques such as ICSI. However, the success rate of ART remains low and fertilisation failure following ICSI is undeniable. ICSI failure is predominantly attributed to OAD and the fundamental role of PLCζ in oocyte activation has been clearly reported, with various studies showing strong association between infertile men diagnosed with OAD and PLCζ deficiency (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Kashir *et al.*, 2013b). However, the precise mechanism of PLCζ in terms of its specific role in male infertility remains unclear, especially in terms of the wide variability in total levels of PLCζ, proportion of sperm exhibiting PLCζ,

and localisation of PLCζ among fertile and infertile men (Kashir *et al.*, 2013b). In the present study, similar variability of PLCζ was observed.

While researchers are focusing upon developing PLCζ as a biological tool to predict the oocyte activation ability of sperm and as a therapeutic agent (Nomikos *et al.*, 2013a), the variability of PLCζ in human sperm is a key factor to consider and may limit the use of PLCζ clinically unless further data is acquired. The heterogeneous nature of sperm and differential PLCζ gene expression could underlie such variation. It is of course possible that the observed variability may be a function of antibody specificity. However, specificity of the PLCζ polyclonal antibody used herein has been proven previously and a mounting body of publications using this antibody have obtained consistent results. However, it would be prudent to develop and utilise a new monoclonal antibody. Use of a monoclonal antibody for subsequent immunofluorescent analysis in human sperm may ultimately confirm or deny our current findings. Moreover, the precise functional role of PLCζ, especially sperm exhibiting differential localisation patterning, could be further investigated via heterologous ICSI and Ca^{2+} release bioassays.

The PLCζ scoring system and PLCζ correlation graphs developed in the present study are likely to represent a beneficial tool for clinical application. The cluster analysis performed earlier in this chapter sets out a novel defined range of PLCζ. The new PLCζ scoring system and the correlative analysis may serve as useful diagnostic and prognostic tools to predict oocyte activation ability. However, fertilisation rate and oocyte activation ability following ART treatment from a larger cohort of fertile and infertile men should be evaluated alongside the PLCζ scoring system and correlation tool to further ascertain whether such tools are beneficial.

Advancing paternal age have been linked with increasing risk of infertility (Belloc *et al.*, 2014), however the effect of increasing male age upon the total levels of PLCζ and OAD have not been reported. Results obtained from the present study did not show any association between increasing male age and the total levels of PLCζ. However, more specific or accurate results should be obtained by increasing the number of samples analysed in future studies.

The identification of an SNP in the PLCζ gene promoter region in two special case study patients was particularly intriguing. Both these patients exhibited different sperm characteristics and infertility history, with one of them exhibiting globozoospermia, a severe sperm morphological condition. Globozoospermia is a complicated genetic condition, where mutation in two genes has been strongly implicated. Thus far, there have been no mutations in the PLCζ gene identified in globozoospermic infertile men. However, the functional aspect of this SNP in the PLCζ gene of this particular patient in relation to PLCζ deficiency, OAD, spermatogenic defects and globozoospermia remains unknown. It will be vital for future studies to perform genetic screening in intronic, exonic and promoter regions of PLCζ gene to identify further SNPs that might present. Compilation of an SNP database for PLCζ could be used to investigate semen quality traits and the expression of PLCζ in patients to evaluate whether SNP can be attributed to infertility in men. It is also crucial to investigate whether such SNPs have a functioned effect or not.

Another important and interesting case study presented herein is patient 6, a vasectomy reversal patient. Sperm from this patient did not only exhibit morphological abnormalities, but also, the total levels of PLCζ were significantly reduced. This patient encountered recurrent ICSI failure. The molecular mechanism underlying such deficiency following vasectomy reversal, or whether such a surgical procedure could affect the expression of PLCζ remains unknown. We are the first to report this finding, although only

one patient has been studied thus far. Consequently, it is difficult to ascertain whether the surgical procedure could affect the expression of PLCζ. A large sample size in future studies will be critical.

In summary, PLCζ plays a fundamental role in oocyte activation and fertilisation following gamete fusion by inducing Ca^{2+} oscillations within the ooplasm to facilitate embryonic development, although the specific functional role within the oocyte still remains undetermined. The potential use of PLCζ as a valuable therapeutic, diagnostic and prognostic tool in ART clinics, especially for ICSI failure cases are important and should be urgently translated to clinics. Application of the present findings in a clinical trial to further ascertains the reliability of the potential tools developed herein is required in order to establish PLCζ as a routine clinical tool to evaluate oocyte activation ability of sperm.

Chapter 5

The potential effect of polyvinylpyrrolidone (PVP) upon Phospholipase C Zeta (PLCζ)

5.1. Introduction

The detrimental effect of cryopreservation upon the total levels of protamine in mouse sperm was clearly apparent in Chapters 2 and 3. However, cryopreservation is not the only laboratory technique involved in the preservation or processing of sperm. There are many other ART techniques utilised in clinics to process, preserve and manipulate gametes and embryos. Sperm processing routinely involves changes in culture conditions, pH, temperature, and specialised techniques such as DGW and sperm immobilisation. While these techniques are frequently used to facilitate selection of the best sperm prior to ART treatment, very little is known about the detrimental effect of such procedures upon sperm structure, DNA integrity and key sperm proteins.

PLCζ is equally important as protamine in determining the success of fertilisation as it plays a fundamental role in oocyte activation following gamete fusion by triggering Ca^{2+} oscillations in the activating oocyte (Amdani *et al.*, 2013). Mounting evidence suggests that PLCζ deficiency in human sperm is strongly linked with male factor infertility (Yoon *et al.*, 2008; Kashir *et al.*, 2013b). Kashir *et al*. (2011a) demonstrated that total levels of PLCζ were significantly reduced in cryopreserved fertile human sperm (Kashir *et al*., 2011a) which was worrying as cryopreservation is commonly used in ART. In fact, these data appeared to suggest that cryopreserved sperm may exhibit reduced oocyte activation ability compared to fresh sperm. This, however, remains to be tested by the injection of such sperm into oocytes with concurrent Ca^{2+} imaging analysis. This chapter extends this earlier study by investigating the effect of sperm immobilisation techniques using PVP upon the expression of PLCζ in human sperm.

The detrimental influence of PVP upon sperm structure, DNA integrity and Ca^{2+} oscillations has been widely reported (Kato & Nagao, 2012; Salian *et al.*, 2012). PVP is a

synthetic viscous solution used to immobilise sperm in order to facilitate sperm handling and selection prior to ICSI. However, the adverse effects of PVP, which may be exerted upon sperm during this procedure is undeniable and may compromise the functional ability of sperm, leading to poor or failure fertilisation. Thus far, it is known that sperm exposed to PVP exhibit structural and plasma membrane damage, nuclear deterioration, and increased DNA fragmentation (Strehler *et al.*, 1998; Salian *et al.*, 2012). Alterations in Ca^{2+} release ability, acrosome reaction, and poor embryonic development following ICSI with sperm exposed to PVP has also been reported (Tesarik & Sousa, 1994; Yanagida *et al.*, 2001). Utilisation of such sperm for ICSI may influence the resultant Ca^{2+} oscillation profile and consequently gene expression, thus affecting embryonic development (Ozil *et al.*, 2006). It remains unknown if the influence of PVP-treated sperm upon Ca^{2+} releasing ability could be due to reduction or abolishment of PLCζ in sperm. Several other sperm immobilisation or pre-treatment techniques such as sonication, repetitive freezing/thawing, and exposure to Triton X-100 have been shown to significantly reduce the total levels of PLCζ in pig boar sperm prior to ICSI (Nakai *et al.*, 2011). It is therefore essential to investigate if sperm exposed to PVP can influence PLCζ content in the sperm head, and thus exert effect upon oocyte activation ability.

The general aim of this chapter was therefore to evaluate and compare the total levels, proportions and localisation pattern of PLCζ between non-PVP and PVP-treated human sperm samples using an established immunofluorescence assay. The experiments described in this chapter are divided into two sections. **Section I** describes a pilot experiment, which examined the potential effect of PVP upon the total levels of PLCζ and the proportion of sperm exhibiting PLCζ in a small number of fertile controls (n=7), infertile patients (n=3) and donors (n=2). These samples are the same as those also used in Chapter 4. **Section II** involved a larger scale, more robust, investigation involving a larger number (n=16) of donor

samples from a Sperm Bank in Germany. This experiment was designed to confirm or refute the findings of the pilot experiment described in **Section I**.

5.1.1 Specific aims of this chapter

Section I

1. To investigate the effect of PVP upon total levels of PLCζ and the proportion of sperm exhibiting PLCζ, and localisation patterning of PLCζ in sperm from fertile controls, infertile patients, and donors.

Section II

2. To compare the viability of sperm from 16 fertile donors incubated with PBS or PVP at 4 different time points and to evaluate whether prolonged incubation of sperm in PBS or PVP can detrimentally affect sperm viability.
3. To evaluate the total levels of PLCζ and the proportion of sperm exhibiting PLCζ in sperm incubated with PBS or PVP for differing periods of time, and to investigate whether the prolonged exposure of sperm to PBS or PVP could exert influence over these important parameters.

5.2 Materials and Methods

5.2.1 Patient selection and ethics

Section I involved a total of 9 fertile controls (including 2 donors) and 3 infertile patients (selected from the 30 human samples recruited for **Section 4.2.1**) along with the two additional donors (Controls 14 and 15) recruited by the OFU. For **Section II**, 16 additional fertile donors samples were obtained from Reprogen GmBh Sperm Bank (Dusseldorf, Germany) with ethical permission approved by the Oxford Tropical Research Ethics Committee (OXTREC Ref: 31-09). These samples were obtained with informed written consent. Samples were cryopreserved and transported in LN_2 to the Oxford laboratory.

Section I

5.2.2 Sperm preparation and PVP treatment

Sperm samples obtained from the OFU were subjected to DGW as described in Chapter 4 (**Section 4.2.2**). Following DGW, the supernatant was discarded and the pellet was re-suspended in 200µl of PBS. This sperm suspension was then split into two aliquots, each of 100µl and both were further centrifuged at 800*g* for 3 minutes. The sperm pellet obtained from one aliquot was immediately fixed with 4% PFA for 10 minutes and then stored in 200µl PBS as described in **Section 4.2.2**. The second sperm pellet was incubated with 50µl of 10% PVP in Ferti-Cult flushing medium (FertiPro, Belgium) for 30 minutes at room temperature. Following incubation, the PVP-treated sample was centrifuged at 800*g* for 3 minutes to remove PVP solution. After one final washing step by centrifugation, the sample was fixed with 100µl 4% PFA for 10 minutes and then stored in 200µl PBS to await analysis.

Section II

5.2.3 Sperm preparation and PVP treatment

Cryopreserved donor semen samples (n=16) were received in cryo-straws and thawed in a 37°C water bath for 1 minute. The semen obtained (300µl) from each donor was then subjected to DGW as described in Chapter 4 (**Section 4.2.2**). Following DGW and prior to fixing with 4% PFA, basic sperm parameters, such as sperm concentration, motile sperm count and progressive motility were assessed using a 20µm Leja slide (Standard Count 2 Chambers Slides, Leja, The Netherlands) and Computer Assisted Sperm Analysis (CASA; Software Version 12.3K, Hamilton Thorne Biosciences, Ceros, UK). The sperm suspension was then centrifuged at 800*g* for 3 minutes and the sperm pellet obtained was re-suspended in 400µl of warm PBS and split into 8 aliquots. These 8 aliquots of sperm suspension were then centrifuged at 800*g* for 3 minutes. Following centrifugation, sperm pellets from 4 aliquots were incubated in 50µl PBS at 37°C for 4 different time points (0, 15, 30 and 45 minutes) respectively. Sperm pellets from the remaining 4 aliquots were treated with 50µl of PVP and incubated at 37°C for the same time periods (0, 15, 30 and 45 minutes). Following treatment with PBS or PVP, these aliquots were washed twice with PBS by centrifugation at 800*g* for 3 minutes. Finally, all (PBS and PVP) treated samples were fixed with 100µl 4% PFA for 10 minutes and then stored in 200µl PBS.

5.2.4 Sperm viability assessment

Sperm viability of each donor sperm treated with PBS or PVP at the 4 different time points were evaluated using Propidium Iodide (PI), (Live/Dead Sperm Viability Kit, Life Technologies, UK), (a well established method to evaluate sperm viability) and Vectashield H-1200 mountant for fluorescence containing DAPI (Vector, UK). 10µl of sperm suspension from each aliquot was first treated with 0.5µl of PI (0.0024mM) for 5 minutes. Following PI treatment, 5µl of sperm treated with PI was added onto glass slides in two replicates. Finally, each sample was mounted with 1µl of DAPI and covered with a 20mm x 20mm glass cover slip. Sperm viability was evaluated using a fluorescence microscope (80*i*, Nikon). Images were acquired at wavelengths of 358nm for blue filter and 535nm for red filter using an exposure of 400ms. Sperm heads stained with PI (red fluorescence) were classified as non-viable sperm, whereas sperm heads stained with DAPI (blue fluorescence) were classified as viable sperm. Approximately 100 sperm were evaluated for each sample.

5.2.5 PLCζ immunofluorescent staining and statistical analysis

Immunofluorescent staining of PLCζ, imaging, quantitative and statistical analysis was performed as described in **Section 4.2.3.2**, **Section 4.2.3.3**, **Section 4.2.3.4**, and **Section 4.2.3.5**, respectively.

5.3 Results: Section I

5.3.1 Comparison of total PLCζ immunofluorescence levels between non-PVP treated and PVP-treated sperm

Quantitative immunofluorescence analysis showed that 9 controls demonstrated a general trend of reduction (by 16% to 99%) in the total levels of PLCζ following PVP treatment compared to non-PVP treated sperm (**Figure 5.1**). Total levels of PLCζ were significantly reduced by 30% to 99% ($P \leq 0.05$; as indicated by the Student's *t*-test) following PVP treatment in five controls (control 1, 5, 6, 8, and 15). The remaining controls exhibited comparable levels of PLCζ between the two treatments.

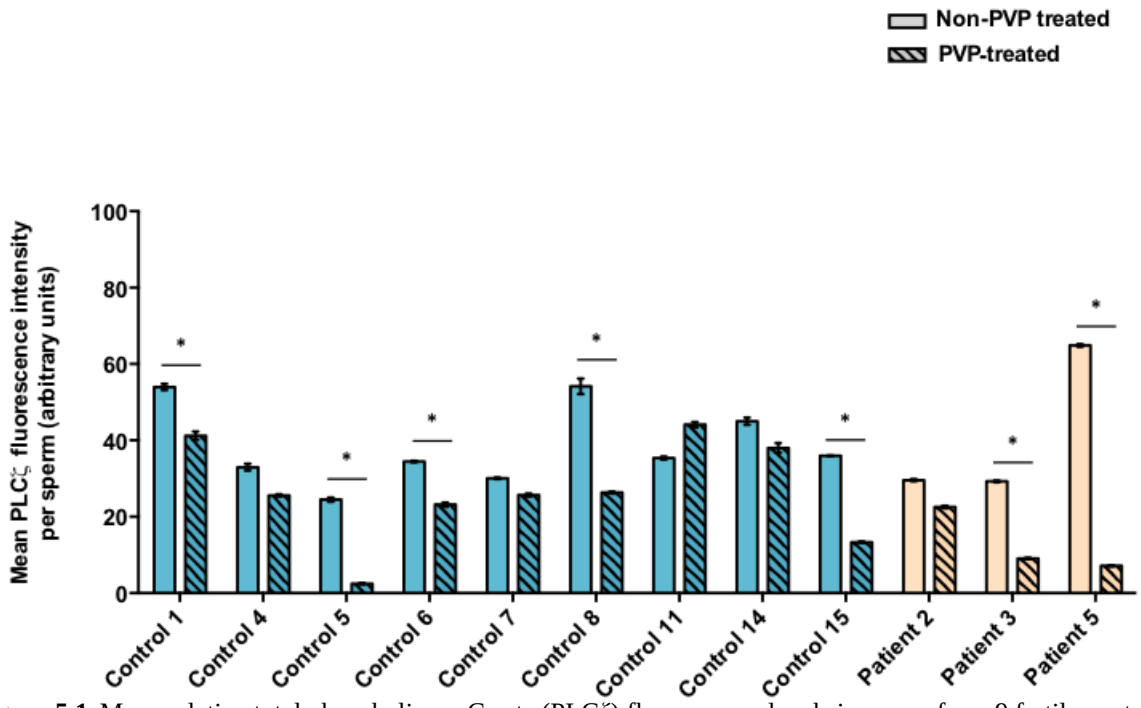


Figure 5.1: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in sperm from 9 fertile controls and 3 infertile patients prior to and following PVP treatment. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisks (*) denote statistically significant differences ($P \leq 0.05$) between non-PVP treated and PVP-treated sperm from each individual control and patient. Data are shown as mean ± SEM

Three infertile patients also showed general reduction (ranging from 24% to 89%) in the total levels of PLCζ in PVP-treated sperm compared to non-PVP treated sperm, although this reduction was only significant in two cases. Patient 3 and 5 showed significant reduction

in the total levels of PLCζ by 67% and 89% ($P \leq 0.05$; as indicated by the Student's *t*-test) respectively following PVP treatment (**Figure 5.1**).

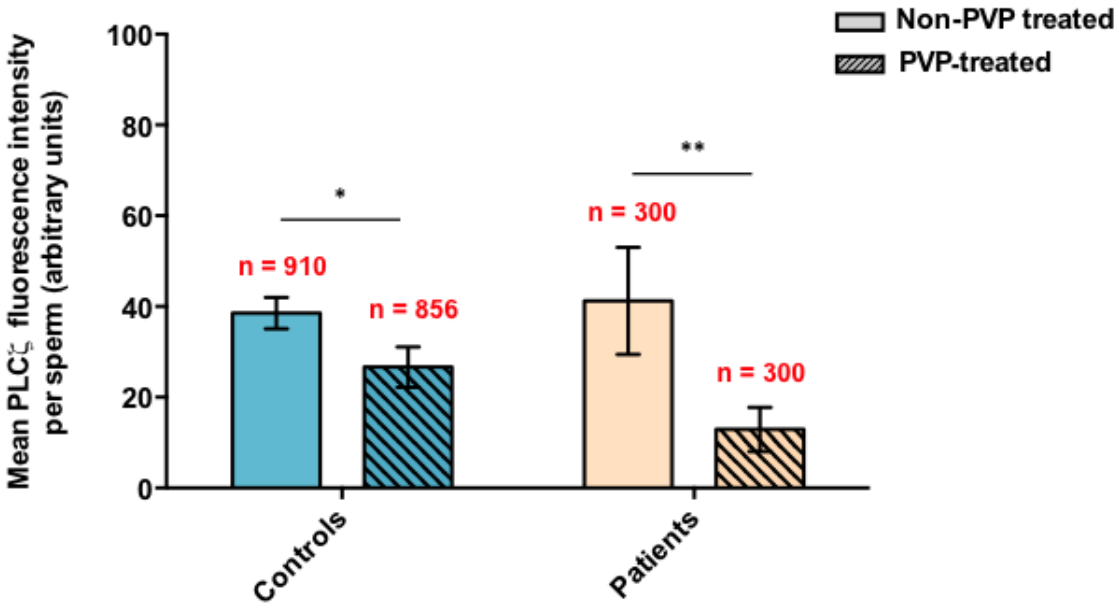


Figure 5.2: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels from 9 fertile controls and 3 infertile patients prior to and following PVP treatment. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. Asterisks (*****, ******) denote statistically significant differences ($P \leq 0.05$) between non-PVP treated and PVP-treated sperm from controls and patients respectively. The numerical values denoted in red font on each histogram represents the total number of sperm analysed. Data are shown as mean ± SEM.

Controls and infertile patients were then compared collectively. Total levels of PLCζ in PVP-treated sperm from all nine controls were significantly reduced by 38% ($P \leq 0.05$; as indicated by the Student's *t*-test) compared to non-PVP treated sperm (n=910) (**Figure 5.2**). A similar trend of significantly reduced ($P \leq 0.05$; as indicated by the Student's *t*-test) levels of PLCζ (by 62.5%) was observed in PVP-treated sperm from all patients compared to non-PVP treated sperm. Among the PVP-treated sperm, total levels of PLCζ in patients were significantly reduced by 50% ($P \leq 0.05$; as indicated by the Student's *t*-test) compared to controls indicating the potential vulnerability of patient sperm to PVP treatment compared to sperm from fertile controls.

5.3.2 Comparison of the proportion of sperm exhibiting PLCζ between non-PVP treated and PVP-treated sperm

The proportion of sperm exhibiting PLCζ in nine controls and three patients following PVP treatment also revealed a general trend of reduction compared to non-PVP treated sperm (**Figure 5.3**). Sperm from controls 1, 5, 6, 8 and 15 showed significant reduction by 22% to 100% ($P \leq 0.05$; as indicated by the Student's *t*-test) in the proportion of sperm exhibiting PLCζ following PVP treatment. Sperm from controls 7 and 11 did not show significant differences in the proportion of sperm exhibiting PLCζ between non-PVP and PVP treated sperm. Interestingly, of the three patients, patients 3 and 5 showed complete absence of PLCζ following PVP treatment compared to non-PVP treated sperm.

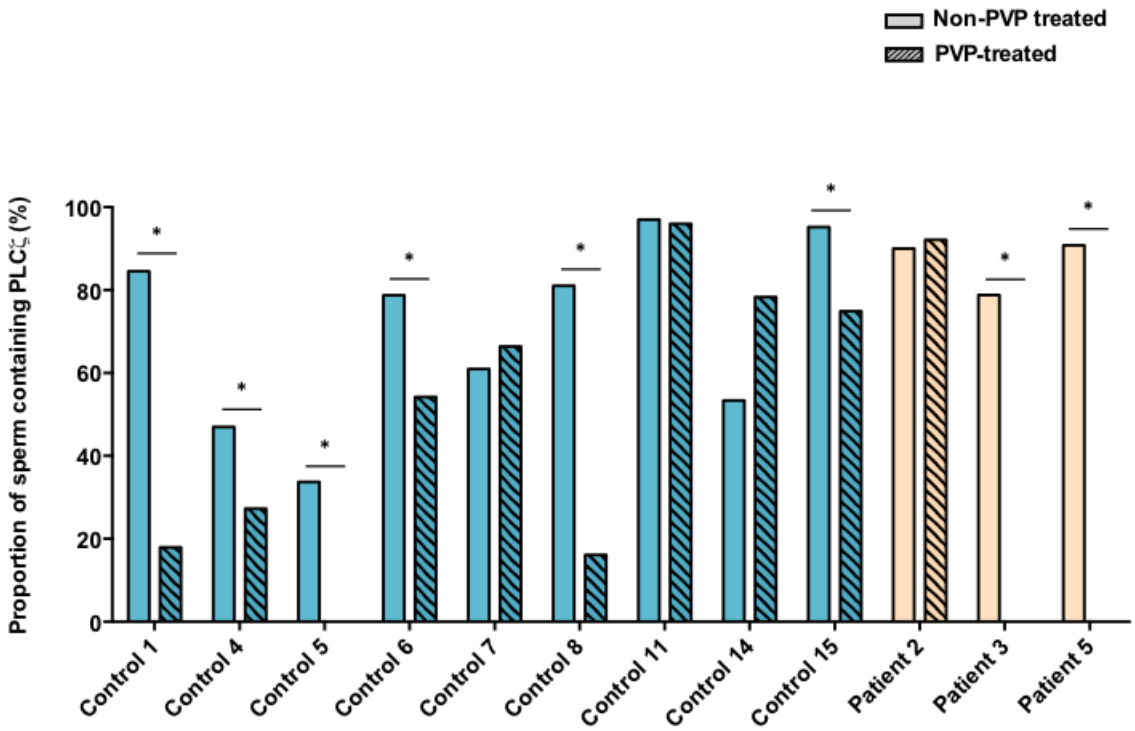


Figure 5.3: Proportion of sperm from 9 fertile controls and 3 infertile patients exhibiting phospholipase C zeta (PLCζ) prior to and following PVP treatment. Number of sperm with PLCζ was determined with the use of ImageJ software. Asterisks (*) denote statistically significant differences ($P \leq 0.05$) between non-PVP treated and PVP-treated sperm from each individual control and patient. Data are shown as percentages.

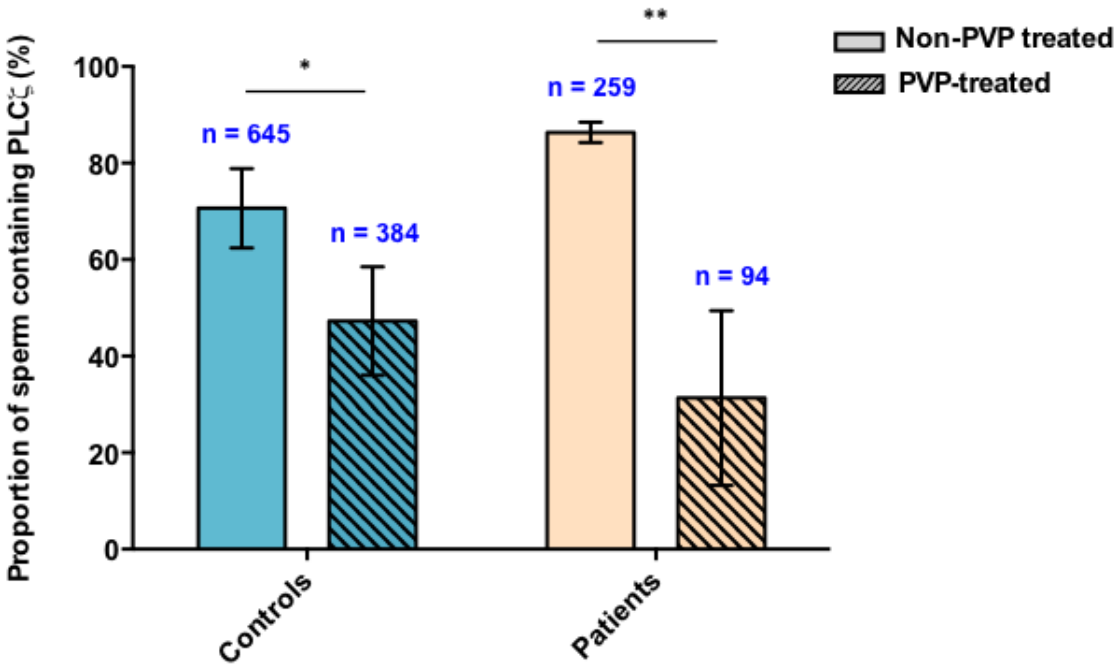


Figure 5.4: Mean proportion of sperm from 9 fertile controls and 3 infertile patients exhibiting phospholipase C zeta (PLCζ) prior to and following PVP treatment. Number of sperm with PLCζ was determined with the use of ImageJ software. Asterisks (*, **) denote statistically significant differences ($P \leq 0.05$) between non-PVP treated and PVP-treated sperm from controls and patients respectively. The numerical values denoted in blue font on each histogram represents the number of sperm containing PLCζ out of total number of sperm analysed. Data are shown as mean ± SEM.

Collectively, proportional analysis of PLCζ among controls showed significant reduction by 33% ($P \leq 0.05$; as indicated by the Student's *t*-test) in the number of sperm exhibiting PLCζ following PVP treatment compared to non-PVP treated sperm. In patients, the proportion of PVP-treated sperm exhibiting PLCζ was significant reduced by 67% ($P \leq 0.05$; as indicated by the Student's *t*-test). This data further indicated that the total number of sperm from the patient group containing PLCζ was significantly reduced by 20%, following PVP treatment as compared to the PVP-treated sperm from the control group (**Figure 5.4**).

5.3.3 Localisation patterning of PLCζ in non-PVP and PVP-treated sperm

The distinctive localisation patterning of PLCζ in sperm from fertile and infertile humans were described in Chapter 4, (**Section 4.3.5**). In this section, the localisation patterning of PLCζ in controls (**Table 5.1**) and patients (**Table 5.2**) were compared between non-PVP and PVP-treated sperm. Earlier studies have shown that PLCζ undergoes changes in localisation patterning following capacitation and the acrosome reaction suggesting the potential involvement of PLCζ in other biochemical functions, aside from oocyte activation alone. Here, the predominant aim was to evaluate whether the exposure of sperm to PVP may induce similar changes in the localisation of PLCζ within the sperm head, which may subsequently influence oocyte activation ability.

Among the controls studied in this chapter, the predominant localisation pattern of PLCζ was observed in equatorial (E), post-acrosomal + equatorial (P+E) and acrosomal + equatorial + post-acrosomal (A+E+P) regions. The total number of sperm exhibiting PLCζ in these regions was significantly reduced following PVP treatment, with (P+E) and (A+E+P) showing significant reduction ($P \leq 0.05$; as indicated by the Student's *t*-test) in PVP-treated sperm (**Figure 5.5**).

Table 5.1: Proportion of sperm exhibiting phospholipase C zeta (PLCζ) localisation patterns in sperm from fertile controls (n=9) prior to and following PVP treatment.

Controls	A (Acrosomal)	P (Post-acrosomal)	E (Equatorial)	A+P	A+E	P+E	A+E+P	None
[- PVP]	1	9	14	1	2	20	24	29
[+PVP]	1	7	13	1	1	15	17	45

[- PVP] = Localisation patterning of PLCζ prior to PVP treatment.
[+PVP] = Localisation patterning of PLCζ following PVP treatment.
Data are shown as percentages.

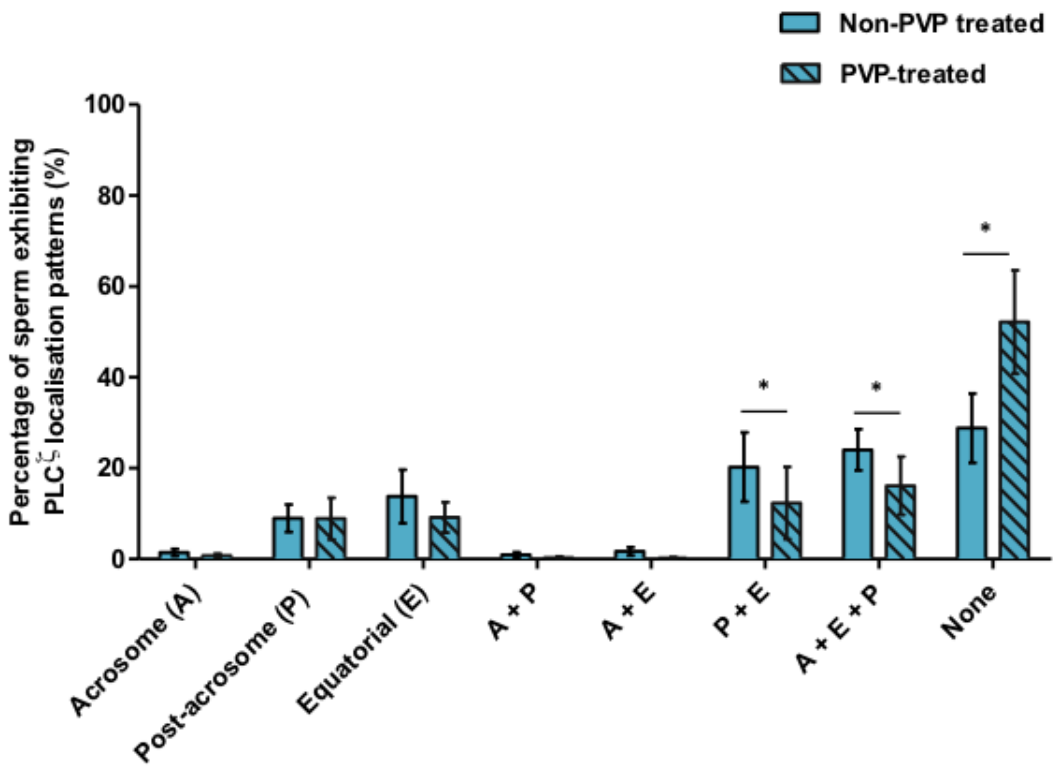


Figure 5.5: Proportion of non-PVP treated and PVP-treated sperm from 9 fertile controls containing phospholipase C zeta (PLCζ) in different localisation patterns. Analysis was performed by ImageJ software. Asterisks (*) denote statistically significant differences ($P \leq 0.05$) between non-PVP and PVP-treated sperm containing PLCζ at P+E, A+E+P localisation patterns and sperm with absence of PLCζ. Data are shown as mean ± SEM.

Table 5.2: Proportion of sperm exhibiting phospholipase C zeta (PLCζ) localisation patterns in sperm from infertile patients (n=3) prior to and following PVP treatment.

Patients	A (Acrosomal)	P (Post-acrosomal)	E (Equatorial)	A+P	A+E	P+E	A+E+P	None
[- PVP]	1	13	2	7	1	21	42	13
[+PVP]	1	20	1	1	0	3	6	68

[- PVP] = Localisation patterning of PLCζ prior to PVP treatment.
[+PVP] = Localisation patterning of PLCζ following PVP treatment.
Data are shown as percentages.

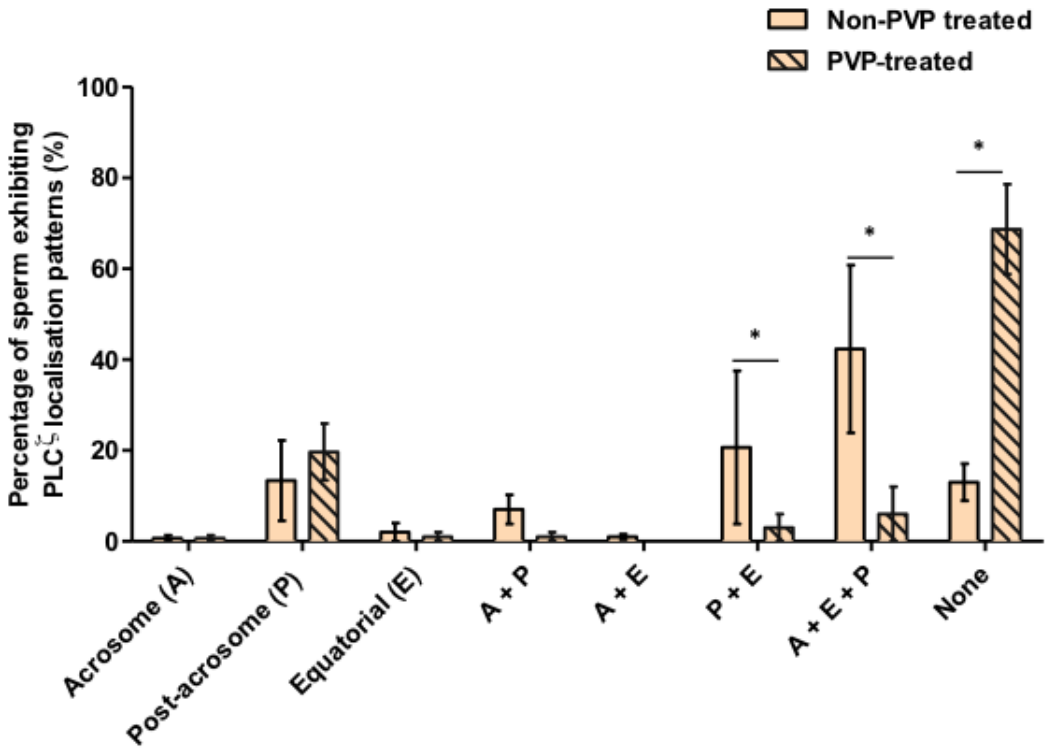


Figure 5.6: Proportion of non-PVP treated and PVP-treated sperm from 3 infertile patients containing phospholipase C zeta (PLCζ) in different localisation patterns. Analysis was performed by ImageJ software. Asterisks (*) denote a statistically significant difference ($P \leq 0.05$) between non-PVP and PVP-treated sperm containing PLCζ at P+E, A+E+P localisation paterns and sperm with absence of PLCζ. Data are shown as mean ± SEM.

The predominant localisation pattern of PLCζ in sperm from patients (P+E, and A+E+P) prior to PVP treatment was significantly reduced ($P \leq 0.05$; as indicated by the Student's *t*-test) following PVP treatment (**Figure 5.6**). Interestingly, localisation of PLCζ in

the post-acrosomal region was increased in PVP-treated sperm compared to non-PVP treated sperm. Localisation patterning of PLCζ for each individual control and patient prior to and following PVP treatment is presented in **Tables 5.3** and **5.4**.

Table 5.3: Percentage of sperm exhibiting phospholipase C zeta (PLCζ) localisation patterns in each individual control prior to and following PVP treatment.

Controls (n=9)	A (Acrosomal)	P (Post-acrosomal)	E (Equatorial)	A+P	A+E	P+E	A+E+P	None
Non-PVP								
1	7.1	2.7	**36.6**	4.4	7.0	14.2	15.9	12.4
4	2.0	7.0	**13.0**	0.0	1.0	7.0	10.0	**60.0**
5	1.0	3.0	0.0	0.0	0.0	8.0	**21.0**	**67.0**
6	3.0	3.0	**49.0**	3.0	2.0	4.0	15.0	21.0
7	0.0	6.0	2.0	0.0	0.0	8.0	**50.0**	**34.0**
8	0.0	27.0	16.7	0.0	5.2	11.5	17.7	22.0
11	0.0	9.0	4.0	1.0	0.0	**67.0**	16.0	3.0
14	0.0	**21.0**	3.0	0.0	0.0	10.0	**29.0**	**37.0**
15	0.0	2.0	**0.0**	0.0	0.0	**52.5**	**41.5**	4.0
PVP								
1	3.5	1.8	8.0	0.9	0.0	0.0	1.8	**84.0**
4	3.0	6.0	9.0	0.0	0.0	3.0	3.0	**76.0**
5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**100.0**
6	0.0	2.0	30.0	0.0	2.0	0.0	21.0	**45.0**
7	0.0	8.0	1.0	0.0	0.0	4.0	**59.0**	28.0
8	0.0	12.0	3.0	0.0	0.0	0.0	1.0	**84.0**
11	0.0	6.0	8.0	2.0	0.0	67.0	13.0	4.0
14	0.0	**44.2**	2.3	0.0	0.0	0.0	30.2	23.3
15	0.0	0.0	**21.0**	0.0	0.0	37.0	16.0	26.0

Numerical value in bold red font indicates the proportion of sperm exhibiting a predominant pattern of PLCζ in sperm from each individual control classified according to their clusters (cluster analysis was performed and presented in Chapter 4). Bold blue font indicates proportion of sperm devoid of PLCζ. Bold black font with yellow highlights indicates increased proportion of specific PLCζ pattern following PVP treatment. Data are shown as percentages.

As shown in **Table 5.3**, several controls showed changes in localisation patterning of PLCζ following PVP treatment. For example, the proportion of sperm exhibiting a post-acrosomal pattern of PLCζ in control 14 increased from 21% to 44.2%. Interestingly, sperm from control 15 did not show the presence of an equatorial population of PLCζ prior to PVP

treatment. However, following PVP treatment, 21% of sperm from control 15 exhibited PLCζ localisation in the equatorial segment of the sperm head.

Table 5.4: Percentage of sperm exhibiting phospholipase C zeta (PLCζ) localisation patterns in each individual patient prior to and following PVP treatment.

Patients (n=3)	A (Acrosomal)	P (Post-acrosomal)	E (Equatorial)	A+P	A+E	P+E	A+E+P	None
Non-PVP								
2	0.0	**4.0**	6.0	6.0	2.0	8.0	**66.0**	8.0
3	0.0	5.0	0.0	13.0	1.0	**54.0**	6.0	21.0
5	2.0	31.0	0.0	2.0	0.0	0.0	**55.0**	10.0
PVP								
2	0.0	**61.0**	3.0	3.0	0.0	9.0	18.0	6.0
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**100.0**
5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**100.0**

Numerical value in bold red font indicates the proportion of sperm exhibiting a predominant pattern of PLCζ in sperm from each individual controls classified according to their clusters (cluster analysis was performed and presented in Chapter 4). Bold blue font indicates proportion of sperm devoid of PLCζ. Bold black font with yellow highlights indicates increased proportion of specific PLCζ pattern following PVP treatment.
Data are shown as percentages.

Patient 2 showed an increased of 57% in the post-acrosomal population of PLCζ localisation patterning following PVP treatment. Prior to PVP treatment, patients 3 and 5 exhibiting a PLCζ predominant localisation pattern in P+E and A+E+P regions respectively. However, following PVP treatment, both patients 3 and 5 were devoid of PLCζ.

Results: Section II

5.3.4 Basic semen data from sixteen donors

Cryopreserved semen samples from donors were first thawed and subjected to DGW as described in **Section 5.2.3**. Sperm assessments were performed using CASA following DGW and CASA data from the sixteen donors are presented in **Table 5.5**. Total motility, progressive motility (a), and total concentration from the 16 donors ranged between 3.7% to 59.3%, 4% to 45%, and 1.2M/mL to 18.3M/mL respectively.

Table 5.5: Mean sperm assessment data obtained from the 16 donors following density gradient washing (DGW) using computer assisted sperm analysis (CASA).

Sperm parameters	Total sperm motility (%)	Progressive motility (a) (%)	Concentration (M/mL)
(n=16)	24.7 ± 4.5	18.5 ± 3.5	5.4 ± 1.7

Data are shown as mean ± SEM.

5.3.5 Comparison of sperm viability between non-PVP treated and PVP-treated sperm from sixteen donors

In general, the proportion of viable sperm in each thawed sample showed a trend of gradual reduction for non-PVP and PVP-treated samples over the four time points analysed (**Figure 5.7**).

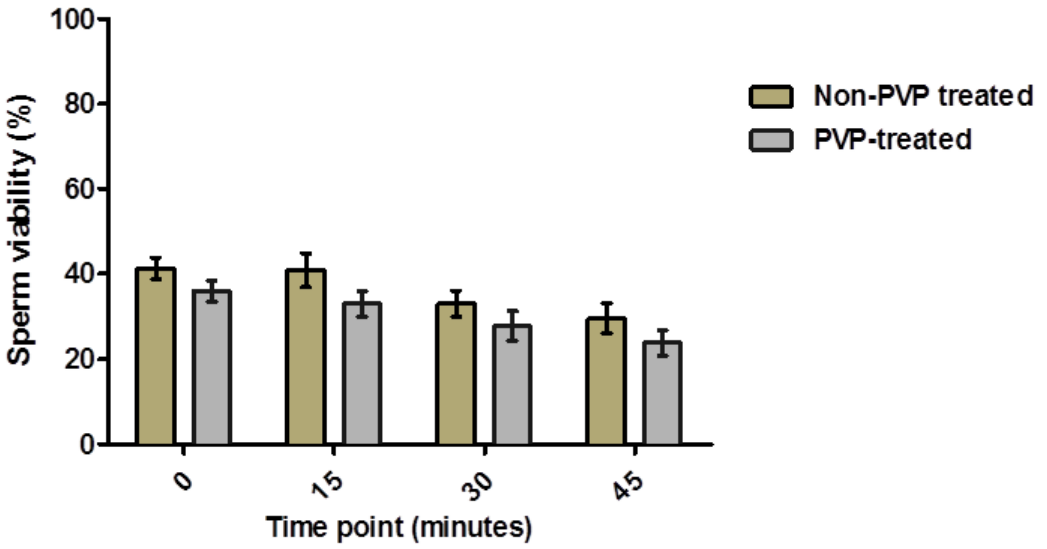


Figure 5.7: Proportion of viable sperm prior to, and following PVP treatment in 16 donor sperm samples. There was no statistically significant difference between non-PVP and PVP-treated sperm from 16 donors. Data are shown as mean ± SEM.

Sperm viability following PVP treatment at each time point was lower (ranging from 12% to 20%) compared to non-PVP treated sperm (**Figure 5.7**) although this was not statistically significant ($P \geq 0.05$; as indicated by repeated measures ANOVA).

5.3.6 Comparison of total PLCζ immunofluorescence levels between non-PVP treated and PVP-treated sperm from sixteen donors

Total levels of PLCζ in non-PVP sperm from 16 donors remained consistent ranging from 32.7 ± 3.6AU to 38.6 ± 4.0AU (**Figure 5.8**) and did not show statistically significant differences ($P \geq 0.05$; as indicated by repeated measures ANOVA) over the four time points analysed.

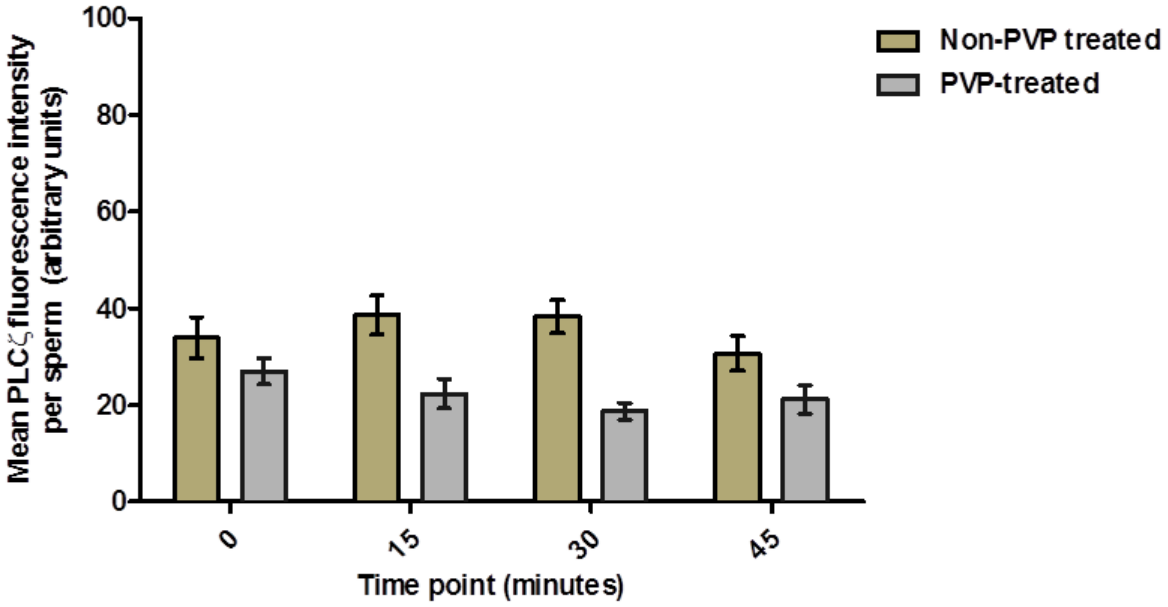


Figure 5.8: Mean relative total phospholipase C zeta (PLCζ) fluorescence levels in sperm from 16 donors prior to, and following PVP treatment. Fluorescence intensity was quantified in arbitrary units (AU) with the use of ImageJ software. There was no statistically significant difference between non-PVP treated and PVP-treated sperm from 16 donors. Data are shown as mean ± SEM.

Similar results were obtained for the PVP-treated sample with total levels of PLCζ ranging from 18.7 ± 1.8AU to 27.0 ± 2.7AU (**Figure 5.8**). This data did not show statistically significant differences ($P \geq 0.05$; as indicated by repeated measures ANOVA) over the four time points. Total levels of PLCζ appeared to be generally lower in PVP-treated sperm compared to non-PVP sperm, but this was not statistically significant ($P \geq 0.05$; as indicated by repeated measures ANOVA).

5.3.7 Comparison of the proportion of sperm exhibiting PLCζ between non-PVP treated and PVP-treated sperm from sixteen donors

The proportion of sperm from 16 donors exhibiting PLCζ showed a general decline for non-PVP treated sperm over the four time points. Similarly, the proportion of sperm exhibiting PLCζ in PVP-treated sperm also showed a general trend of reduction across the four time points (**Figure 5.9**). However, these reductions were not statistically significant ($P \geq 0.05$; as indicated by repeated measures ANOVA).

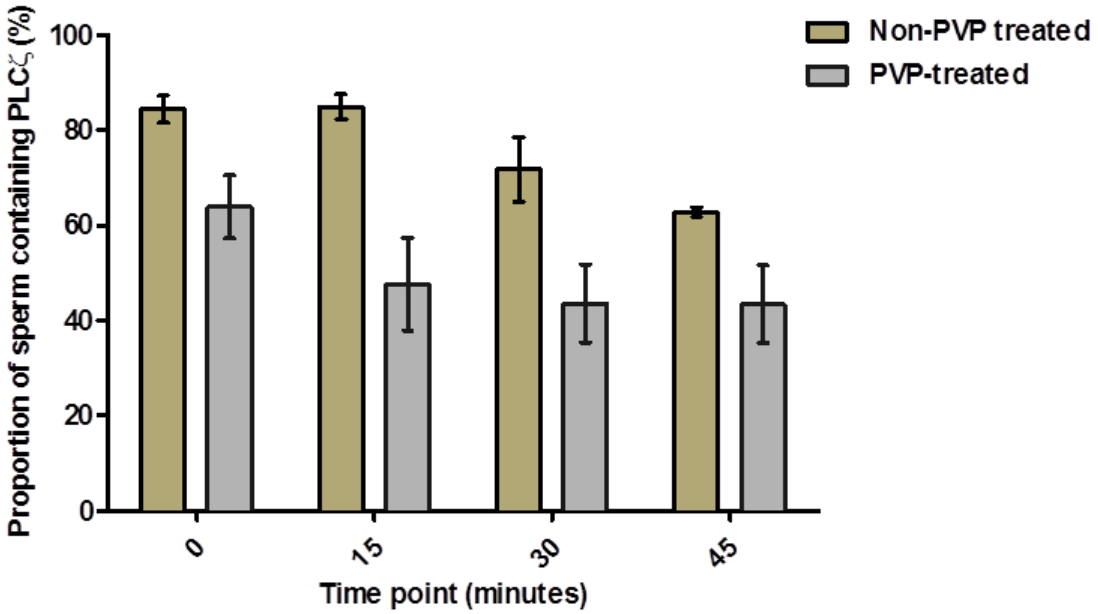


Figure 5.9: Proportion of sperm from 16 donors exhibiting phospholipase C zeta (PLCζ) prior to, and following PVP treatment. Number of sperm with PLCζ was determined with the use of ImageJ software. There was no statistically significant difference between non-PVP treated and PVP-treated sperm from 16 donors. Data are shown as mean ± SEM.

Comparative analysis performed between non-PVP and PVP-treated sperm at each time point revealed a lower proportion of sperm exhibiting PLCζ in PVP-treated sperm. As incubation time increased, the proportion of sperm containing PLCζ was lower (by 19% to 36%) following PVP treatment compared to non-PVP treated sperm (**Figure 5.9**). However, this difference was not statistically significant ($P \geq 0.05$; as indicated by repeated measures ANOVA).

5.4 Summary of results

Section I

1. Total levels of PLCζ and the proportion of sperm containing PLCζ were significantly reduced in PVP-treated sperm from both controls and patients. Importantly, sperm from some individuals appeared devoid of PLCζ following PVP treatment.
2. The total levels and proportion of sperm exhibiting PLCζ in PVP-treated sperm from patients were significantly lower compared to PVP-treated sperm from controls, suggesting that patient sperm were more susceptible to PVP treatment.
3. Reduction in the total levels and proportion of sperm exhibiting PLCζ in PVP-treated sperm varied widely, indicating that the susceptibility of sperm to PVP treatment may depend upon the initial concentration of PLCζ protein in an individual sperm.
4. The distinct localisation of PLCζ in sperm prior to PVP treatment showed significant reduction in the predominant original region of PLCζ, and in some cases this localisation patterning appeared to undergo alteration following PVP treatment.

Section II

5. Sperm viability showed a general trend of reduction with increasing incubation time for both non-PVP treated (PBS-incubated sperm) and PVP-treated sperm samples from sixteen donors. Although viability of PVP-treated sperm appeared to be lower compared to non-PVP treated sperm, however, these data did not show statistically significant differences.
6. Total levels of PLCζ also showed a general trend of reduction as incubation time increased in both non-PVP treated (PBS-incubated sperm) and PVP-treated sperm from sixteen donors. Total levels of PLCζ were lower in PVP-treated sperm compared to non-PVP treated (PBS-incubated sperm) at each time point, but these differences were not statistically significant.
7. The proportion of sperm exhibiting PLCζ generally decreased steadily with increasing incubation time in PBS and PVP. As compared to non-PVP treated (PBS-incubated sperm), the proportion of sperm containing PLCζ in PVP-treated sperm appeared lower. However, this was not statistically significant.

5.5 Discussion

Various sperm preparations, pre-treatments and immobilisation techniques are deployed in ART in order to select the best sperm with optimal fertilising ability (Said *et al* 2011, Parmegianni *et al* 2014). Such techniques allow embryologist to avoid sperm exhibiting poor motility, DNA integrity (Parmegiani *et al*., 2010) and oocyte activation ability (Vanden Meerschaut *et al.*, 2013). Evidence indicates that sperm immobilisation techniques during ICSI can cause detrimental effect upon sperm structure, membrane integrity, the nucleus, DNA integrity and the acrosome (Gomez-Torres *et al.*, 2007; Kato & Nagao, 2012; Salian *et al.*, 2012). Although some believe that sperm immobilisation or pre-treatment is required to promote acrosomal and plasma membrane disruption in order to facilitate successful fertilisation following ICSI (Dozortsev *et al.*, 1995; Velaers *et al.*, 2012), such techniques also intend to help sperm to undergo nuclear decondensation following fertilisation to form pro-nuclei (Velaers *et al.*, 2012). However, questions have been raised in terms of the precise extent to which sperm structural disruption is required for successful ICSI (Velaers *et al*., 2012). Moreover, whether such techniques can detrimentally affect sperm proteins essential for fertilisation remains undetermined. While ICSI remains a highly reliable treatment in the field of ART with a mean fertilisation rate of 70%-80%, ICSI failure still occurs and is predominantly associated with OAD (Vanden Meerschaut *et al.*, 2013). The link between OAD and PLCζ deficiency in human sperm is well known and has been described at length elsewhere in this thesis (Yoon *et al.*, 2008; Heytens *et al.*, 2009).

While the fundamental role of PLCζ in the initiation of oocyte activation is well established, it remains unknown as to whether sperm pre-treatment or immobilisation techniques can exert influence upon the levels of PLCζ within human sperm. Interestingly, pre-treatment of boar sperm to promote plasma membrane disruption significantly reduced

the amount of PLCζ and reduced fertilisation rate following ICSI (Nakai *et al.*, 2011). Additionally, Binh *et al*. (2009) reported that liquid preservation of boar semen at different time points and temperatures may influence sperm oocyte activation rate, pro-nuclei formation and embryonic development (Binh *et al.*, 2009). In addition, a previous study from the Coward Laboratory indicated that total levels of PLCζ were significantly reduced in cryopreserved sperm from fertile controls Kashir *et al*. (2011a).

The detrimental effect of sperm immobilisation techniques using PVP upon sperm has been widely reported. The use of PVP tends to induce acrosome exocytosis via cellular membrane disruption and led to the liberation of oocyte activation factors (Kato & Nagao, 2009). PLCζ localisation patterns are known to undergo dynamic changes during capacitation and the acrosome reaction (Grasa *et al.*, 2008; Young *et al.*, 2009). Findings from these studies suggest that PVP may also detrimentally disrupt the sperm membrane and acrosome, consequently affecting the expression and localisation of PLCζ within the sperm head and potentially interfering with Ca^{2+} release and gene expression.

Therefore, the main objective of this chapter was to investigate the effect of PVP upon the levels of PLCζ in human sperm using an established quantitative immunofluorescence assay. In addition, experiments aimed to investigate potential changes in localisation pattern and the proportion of sperm exhibiting PLCζ. Knowledge acquired may allow improvements or modifications to current ICSI techniques to enhance ART success.

Section I: Pilot experiment

Data generally demonstrated a reduction in the total immunofluorescence levels of PLCζ in PVP-treated sperm compared to non-PVP treated sperm in fertile controls. Sperm treated with PVP from nine fertile controls (including two donors) showed a reduction in the total levels of PLCζ following PVP treatment with five controls demonstrating significant reduction. Among the three patients, the mean relative fluorescence levels and proportion of sperm exhibiting PLCζ in sperm from two patients (patient 3 and 5) demonstrated significant reduction compared to non-PVP sperm.

A higher proportion of sperm exhibited PLCζ in non-PVP treated sperm compared to PVP-treated sperm. More than 80% of the non-PVP treated sperm (analysed collectively) exhibited PLCζ. In contrast, marked reductions in the proportion of sperm containing PLCζ were observed in PVP-treated sperm. Critically, one fertile control and two infertile patients showed complete absence of PLCζ following PVP treatment

Total immunofluorescence levels and proportional analysis of sperm from all nine fertile controls (including two donors) and three patients were quantified and analysed. Collectively, both fertile controls and patients indicated a significant reduction of PLCζ protein in PVP-treated sperm compared to non-PVP treated sperm. Hence, these findings may suggest that the use of PVP causes deleterious effect to sperm consequently leading to changes in PLCζ content, with consequential effect upon oocyte activation ability.

Interestingly, the total levels of PLCζ and the proportion of sperm exhibiting PLCζ, were significantly reduced in PVP-treated sperm from patients as compared to sperm from fertile controls. This may therefore suggest that sperm from patients may be more susceptible to the deleterious effects of PVP than controls. Current data also demonstrated that the proportion of sperm from control 5, patient 3 and 5 exhibited complete absence of PLCζ

following PVP treatment. Previous publications, and the findings presented in this thesis, showed that the total levels of PLCζ vary significantly between sperm from different individuals and within an individual ejaculate (Grasa *et al.*, 2008; Kashir *et al.*, 2013b). The data obtained in this chapter imply that sperm from different individuals may be susceptible to the deleterious effect of PVP in a variable manner, and that the extent of such effect may depend upon the amount of PLCζ protein present prior to treatment.

Analysis of PLCζ localisation patterns during the experiment described in this chapter showed significant variation in sperm from both fertile controls and infertile patients, as previously observed by Grasa *et al*. (2008) and Kashir *et al*. (2013b). While these two earlier papers demonstrated (E) and (P+E) as the predominant localisation patterns (Grasa *et al.*, 2008; Kashir *et al.*, 2013b), the findings of this chapter revealed that sperm from either non-PVP or PVP-treated sperm from fertile controls and patients demonstrated (A+E+P) and (P+E) PLCζ localisation patterns as the predominant population of PLCζ. Significant changes in the PLCζ localisation patterning were observed in sperm from several fertile controls and in one infertile patient. Prior to PVP treatment, control 14 and control 15 exhibited predominant PLCζ localisation patterning in (A+E+P) and [(P+E) + (A+E+P)] regions respectively. The proportion of sperm exhibiting PLCζ localisation patterns in these predominant regions of these controls was reduced significantly following PVP treatment. Patient 2 initially exhibited (A+E+P) as the predominant localisation pattern in non-PVP treated sperm. However, following PVP treatment, the proportion of sperm exhibiting (P) localisation of PLCζ increased while (A+E+P) localisation was reduced compared to non-PVP treated sperm. This observation agrees with the findings by Grasa *et al.* (2008) and may suggest that PVP may cause biochemical changes in sperm as previously reported in capacitated and acrosome reacted sperm, leading to changes in overall PLCζ content (Grasa *et al.*, 2008). Furthermore, this study indicated that PVP might be harmful enough to

completely abolish PLCζ content, in some cases as seen in sperm incubated with PVP from control 5, patient 3 and patient 5.

Several studies have shown iatrogenic damage arising from sperm pre-treatment, manipulation, preservation, and cryopreservation, upon the total levels of PLCζ in sperm from humans and other mammalian species (Binh *et al.*, 2009; Heytens *et al.*, 2009; Nakai *et al.*, 2011). Findings from these studies clearly indicated a significant reduction in the total levels of PLCζ following such treatments. In line with these studies, the current data is the first to investigate the detrimental effects of PVP in human sperm, and revealed significant reduction in the total levels of PLCζ following PVP incubation. Data also suggested that it would be also interesting to examine and compare Ca^{2+} oscillation profiles in non-PVP and PVP-treated human sperm. This would further confirm that deterioration of PLCζ in PVP-treated sperm might elicit abnormal Ca^{2+} release patterns and thus compromise oocyte activation mechanisms.

Section II: Larger scale study

This experiment aimed to utilise a larger number of samples to confirm or refute the findings of **Section I**. Sperm from 16 donors incubated with PBS or PVP showed a gradual reduction in viability as incubation time increased. In general, viability of sperm incubated with PVP was lower at each time point compared to those incubated with PBS. However, these differences were not statistically significant.

PVP, a high molecular weight viscous solution, may protect the plasma membrane of sperm, preserving membrane integrity, thus helping to retain viability. For example, one particular study by Dozorstev *et al*. (1995) demonstrated that human sperm exposed to high concentrations of PVP (eg. 10%) had a higher degree of intact sperm plasma membrane compared to sperm incubated with just 2.5% PVP. Moreover, fertilisation rates were higher

following ICSI with sperm incubated with 2.5% PVP compared to 10% (Dozortsev *et al.*, 1995). The lower fertilisation rate observed in this previous study may also be related to PVP retention in the oocyte, which may influence growth of the embryo (Jean *et al.*, 2001; Kato & Nagao, 2009). This study did not only show the protective effect of higher concentrations of PVP, but also demonstrated that PVP prevents sperm nuclear decondensation, which is essential for pronuclear formation following fertilisation (Dozortsev *et al.*, 1995).

Most ART clinics use either 8% or 10% PVP to immobilise sperm prior to ICSI (Kato & Nagao, 2012). A questionable point raised by Dozorstev *et al*. (1995) was that ICSI failure may be related to failure of the sperm nucleus to decondense following injection into oocytes due to the protective effect of 10% PVP, which may have remained coated to the sperm. Sperm immobilisation during ICSI is thought to be required to promote sperm plasma membrane damage and sperm nuclear decondensation in order to facilitate fertilisation (Dozortsev *et al.*, 1995; Nakai *et al.*, 2011). Although the degree of sperm plasma membrane damage required for ICSI remains undetermined (Velaers *et al.*, 2012), the use of PVP has been shown to cause ultrastructure damage to the sperm plasma membrane, nuclei, DNA integrity and influence both the acrosome reaction and Ca^{2+} release profile (Dozortsev *et al.*, 1995; Strehler *et al.*, 1998; Yanagida *et al.*, 2001; Kato & Nagao, 2009; Kato & Nagao, 2012; Salian *et al.*, 2012). However, since there were no functional studies performed in the present study, very little is known of fertilisation outcome such as oocyte activation and pronuclear formation following ICSI with human sperm exposed to PVP at different time points.

The detrimental effect of cryopreservation upon basic sperm parameters, sperm viability, DNA integrity and sperm proteins are clearly evident (Zribi *et al.*, 2010; Gadea *et al.*, 2011; Di Santo *et al.*, 2012; Zribi *et al.*, 2012; Xiaoli *et al.*, 2013). In particular, frozen/thawed human sperm incubated with PVP showed higher levels of DNA fragmentation compared to fresh sperm (Salian *et al.*, 2012). Limitations to the current study

are that the basic sperm parameter and viability assessment data for the donor semen prior to cryopreservation in Germany were not available. Moreover, sperm parameters and DNA integrity were not evaluated following incubation with PBS or PVP at each time point due to low volume and concentration of the samples. Future studies may need to take these limitations into consideration and to perform functional experiments such as ICSI with sperm exposed to PBS or PVP at increasing time points to fully evaluate the fertilisation ability of such sperm.

Mean total immunofluorescence levels of PLCζ and the proportion of sperm exhibiting PLCζ in sperm from 16 donors incubated in PBS or PVP appeard to show a gradual decrease with incubation time. Sperm incubated with PVP showed lower levels of PLCζ and proportion of sperm exhibiting PLCζ, and suggested that PVP may affect levels of PLCζ when sperm are incubated for longer periods of time. This finding concurs with the data obtained from **Section I**. However, in **Section II**, such changes were not statistically significant. Reduction in sperm membrane integrity can cause liberation or leakage of sperm proteins (Lasso *et al.*, 1994), such as PLCζ within the sperm head which may have led to the findings described herein. It is highly likely that the incubation of sperm in sperm preparation media (such as swim-up medium or DGW buffers) also can exert adverse effects upon DNA integrity (Matsuura *et al.*, 2010; Enciso *et al.*, 2011), especially when incubation time is prolonged. Although PBS solution is not a synthetic viscous solution, like PVP, it is well known that longer exposure of sperm to wash mediums, or PBS solution, can increase oxidative stress and the formation of ROS and detrimentally affect sperm integrity (Hughes *et al.*, 1998; Tavalaee *et al.*, 2012a), and may also cause reduction in the total levels of PLCζ (unpublished data from the Coward Laboratory).

Different sperm immobilisation and treatment techniques can exert various degrees of effect upon sperm structure, membrane integrity and concentration of PLCζ. For example,

Nakai *et al*. (2011) used three different sperm treatment techniques such as sonication, incubation with Triton X-100 solution, and repetitive freeze/thaw to promote sperm plasma membrane damage and to facilitate sperm decondensation in order to increase fertilisation rates in pigs following ICSI (Nakai *et al.*, 2011). The aim of this study by Nakai *et al*. (2011) was to determine the effects of these three techniques upon the total levels of PLCζ. Notably, these sperm pre-treatment techniques resulted in a reduction in the total levels of PLCζ with repetitive freeze/thawing representing the most detrimental technique (Nakai *et al.*, 2011). In line with Nakai *et al*. (2011), the results of the present study showed that human sperm incubated with PVP at different time points showed different levels of effect upon total levels of PLCζ and the proportion of sperm containing PLCζ.

It is interesting to note that PVP exposure led to statistically significant changes in PLCζ in **Section I** but failed to show significance in the larger scale study reported in **Section II**. The use of donor semen samples, which were cryopreserved and thawed prior to analysis in **Section II** may have influenced the data obtained. Cryopreservation can significantly reduce the total levels of PLCζ in fertile control human sperm compared to non-cryopreserved sperm Kashir *et al*. (2011a). In the current study, the initial concentrations of PLCζ in these donor sperm samples were not known prior to cryopreservation. It is highly possible that sperm from these donors may have initially exhibited higher levels of PLCζ prior to cryopreservation, and that following thawing and subsequent treatment with PBS or PVP may have significantly reduced the total levels of PLCζ. However comparative analysis between the original fresh sample and the cryopreserved aliquots was not possible. These very different conclusions are interesting and clearly require further investigation. Whether the use of donor samples that had been cryopreserved for **Section II** compromised analysis is not known and needs to be addressed. The pilot data from **Section I** however, do appear to indicate a link between PVP treatment and detrimental alterations in PLCζ content.

Of great interest was the fact that the initial experiments in **Section I** showed that sperm from infertile patients appeared to be more vulnerable to PVP treatment than sperm from fertile controls, since the reduction in total levels of PLCζ and the proportion of sperm exhibiting PLCζ was greater in infertile patients. In **Section II**, it appeared that sperm incubated with PBS or PVP can incur effects upon the total levels of PLCζ and the proportion of sperm exhibiting PLCζ, with PVP showing greater effect than PBS. These changes, however, were not statistically significant and appeared to indicate that in donor sperm, at least PVP does not have significant effect upon PLCζ.

5.6: Summary and future directions

The findings of this chapter clearly suggest that PVP may exert adverse effect upon the total levels of PLCζ and proportion of sperm exhibiting PLCζ in human sperm. Moreover, increasing incubation of sperm with PVP or PBS appeared to reduce total levels of PLCζ and the proportion of sperm exhibiting PLCζ, although without statistical significance. Findings may suggest that procedures involving prolonged exposure of sperm to PVP could alter intracellular Ca^{2+} release leading to oocyte activation deficiency. Findings from **Section I** yielded statistically significant differences in PLCζ content following PVP treatment. However, findings from **Section II** did not show statistical significance and therefore suggested that PVP does not cause effect, at least in thawed sperm from fertile donors. It is imperative that this discrepancy is investigated further. As indicated in Chapter 4, the total number of sperm used for quantification in our immunofluorescent assay may influence the statistically significance of the data obtained. In **Section II** of this chapter, the mean sperm concentration of the donor samples obtained and the number of sperm recovered from each PBS or PVP treatment for analysis was low. Consequently, in some samples, the number of

sperm used for PLCζ immunofluorescent analysis was low (10-30 sperm) and this might have influenced statistical significance.

However, the current study is obviously lacking in functional studies to expand these potentially interesting findings. Future studies should first utilise a larger number of sperm samples focusing particularly on cryopreserved samples versus fresh samples. Secondly, since PLCζ activates oocytes via intracellular Ca^{2+} release following gamete fusion, it would be prudent to investigate the Ca^{2+} release ability of non-PVP and PVP-treated human sperm by heterologous ICSI. Moreover, it is also important to evaluate fertilisation rates and sperm decondensation rates of sperm incubated with PVP or PBS. To our knowledge, thus far, only one study has examined the protective effect of PVP upon the sperm plasma membrane and that sperm exposed to higher concentrations of PVP have lower fertilisation rates (Dozortsev *et al.*, 1995). Therefore, it is essential for future studies to investigate the effect of different concentrations of PVP, PBS, fertilising medium, or hyaluronic acid (HA), upon sperm viability, DNA integrity and PLCζ along with functional studies such as heterologous ICSI to evaluate oocyte activation ability and fertilisation rates. This may provide a better understanding of how the concentration, content and nature of a medium could potentially influence the functional ability of sperm. A preliminary study from the Coward Laboratory has shown that human sperm incubated with HA medium exhibited higher levels of PLCζ compared to sperm incubated with PVP (data not shown). This finding does not only suggest the detrimental effect of PVP but also recommends the use of HA, a physiological solution to facilitate sperm selection during ICSI, as a potential alternative to PVP. Several other studies have also shown the advantage of using HA compared to PVP (Parmegiani *et al*., 2010).

In summary, PVP is known to cause detrimental effects upon sperm structure, plasma membrane integrity and DNA integrity (Dozortsev *et al.*, 1995; Strehler *et al.*, 1998; Salian *et al.*, 2012; Parmegiani *et al.*, 2014). Although, the effect of PVP upon Ca^{2+} releasing ability,

acrosome reaction and fertilisation rates are evident (Yanagida *et al.*, 2001; Kato & Nagao, 2012), it has not yet been ascertained as to whether such effects could be associated with reduced levels of PLCζ. To our knowledge, this is the first study to perform such an investigation in sperm from fertile and infertile humans and resultant data, at least in part appears to suggest a possible effect indicating the need for further study and development of potential alternatives.

Chapter 6

General conclusions

6.0 General conclusions

ART has now contributed to the birth of 5 million babies worldwide, albeit with a relatively low rate of success. Laboratory procedures have developed enormously in ART in an attempt to improve success rates. Many sophisticated techniques and treatments have been developed to facilitate the selection of gametes and embryos of optimal integrity and quality. Over recent years, a wide range of new methodologies have been integrated in many clinics. These include array comparative genomic hybridization (aCGH) (an embryo aneuploidy assessment technique), a variety of new embryo culture media (sequential or continuous media), time lapse imaging of embryo development (Embryoscope) and "EmbryoGlue" (a medium containing hyaluronan to promote embryo implantation). Such efforts provide additional tools not to only facilitate selection of the best quality embryos in terms of genetic or structural integrity but also to promote successful implantation (Personal Communication, Dr Karen Turner). In addition, a variety of advanced sperm selection techniques have been developed over recent times. These include MSOME, magnetic activated cell-sorting system (MACs), HA medium (Said & Land, 2011; Parmegiani *et al.*, 2014) and RAMAN spectroscopy (named after Sir C.V. Raman) (Mallidis *et al.*, 2011). These techniques are being increasingly used to help select sperm with the best morphology and genetic integrity. The use of MSOME and RAMAN spectroscopy is particularly useful in selecting the best individual sperm for ICSI (Mallidis *et al.*, 2011; Sermondade *et al.*, 2011). However, such advanced technology is not yet commonplace and can incur addition cost and manpower.

The poor success rate of ART can be attributed to a variety of reasons (**Chapter 1**, **Section 1.1**). However, it is known that routine gamete manipulation, pre-treatment and preservation techniques such as sequential embryo culture media, conventional embryo incubators, cryopreservation, DGW, and sperm immobilisation using PVP, can adversely

affect the quality or integrity of essential proteins leading to fertilisation failure. Therefore there is an urgent need to investigate exactly how such techniques can influence gamete quality and thus indicate potential ways in which techniques can be modified, improved, or even avoided.

The major aim of this thesis therefore was to investigate whether the use of routine clinical sperm preservation and manipulation techniques such as cryopreservation and sperm immobilisation by PVP could adversely affect two key sperm proteins and thus compromise functional ability leading to fertilisation failure. Experiments predominantly focused upon the abundance and localisation of protamine and PLCζ prior to and following cryopreservation or PVP treatment, respectively. Other experiments aimed to develop a novel immunofluorescent detection assay for protamine quantification in sperm, to develop a scoring system for PLCζ in human sperm as a novel prognostic indication of oocyte activation ability and to enhance our critical understanding of PLCζ by expanding a patient screening study. Collectively, the outcome of these hypotheses clearly suggested the detrimental effect of cryopreservation, and to a lesser extent immobilisation with PVP, upon the content of protamine and PLCζ in mouse and human sperm respectively. Moreover, the novel protamine quantitative immunofluorescent assay developed herein showed potential for clinical utilisation to determine DNA integrity and fertilisation ability of sperm. Notably, the PLCζ scoring system and PLCζ correlation model with fertilisation rates generated herein indicated a clear potential use as a clinical diagnostic and prognostic biomarkers.

In Chapters 2 and 3, I investigated the effect of cryopreservation upon the total level and localisation patterning of P1, P2, and the P1:P2 ratio in a mouse model using a novel immunofluorescent assay and an established acid-urea gel electrophoresis technique. Cryopreservation was selected for this investigation because this represents a commonplace technique in ART clinics for the long-term storage of patient sperm (Jain & Paulson, 2006;

Di Santo *et al.*, 2012). Cryopreservation is mainly used to preserve sperm from donors, sperm retrieved from testicular biopsies to prevent repetitive biopsy, and from cancer patients prior to chemotherapy or radiotherapy (Di Santo *et al.*, 2012). While the advantages of sperm cryopreservation are well known, it is also apparent that this technique confers risk upon basic sperm parameters, structure, DNA integrity, and sperm proteins which play critical roles in oocyte activation and early embryogenesis (Kashir *et al.*, 2011a; Valcarce *et al.*, 2013; Wang *et al.*, 2013). Protamine is an essential nuclear protein involved in determining the hydrodynamic structure of sperm, along with sperm motility, morphology, concentration and DNA integrity. Collectively, these features dictate progressive motility and fertilisation ability (Francis *et al.*, 2014). Protamine was therefore selected as a key sperm protein with which to study the potential detrimental effect of cryopreservation. Moreover, protamine deficiency and alterations in P1:P2 have been related with male infertility previously (Oliva, 2006). Findings from the current study would provide a better understanding of how external factors could cause protamine deficiency and whether protamine quantification could be used in ART clinics as a diagnostic or prognostic assay.

In line with these hypotheses, results from Chapters 2 and 3 clearly demonstrated the deleterious effect of cryopreservation upon total levels of protamine in mouse sperm implying detrimental effect upon DNA integrity. P1 and P2 were both significantly reduced following cryopreservation. The novel immunofluorescent assay developed in Chapter 2 shows promise as a potential clinical tool and may be equally useful in cases involving cryopreservation. Deficiency of protamine, and consequential effect upon DNA integrity, could cause premature sperm decondensation, leading to fertilisation failure. Fertilisation failure is not always inevitable for such sperm (Sakkas & Alvarez, 2010). However successful fertilisation with such sperm may exert long-term genetic and epigenetic risk in the offspring (Fernandez-Gonzalez *et al.*, 2008; Fedder *et al.*, 2013; Sandin *et al.*, 2013a).

Utilisation of the protamine immunofluorescent assay could potentially predict DNA integrity and fertilisation ability and avoid the risk of genetic or epigenetic defects.

The P1:P2 ratio of cryopreserved mouse sperm determined in this thesis did not show significant differences compared to fresh sperm, probably because the reduction of P1 and P2 were identical following cryopreservation. This may indicate that P1:P2 ratio evaluation to predict fertilisation ability of sperm due to external affect may not be a useful assessment. These data, generated from the mouse model, indicate that the P1:P2 ratio may not be a good marker to predict infertility in human sperm, despite the fact that several studies have suggested the converse. Currently the P1:P2 ratio in fertile human sperm seems to fall between a wide range of 0.53-1.43 (Nanassy *et al.*, 2011), which contradicts the initial suggested ratio of 0.8-1.2 published in previous literature (Carrell *et al.*, 2007). Published data clearly shows wide variation in P1:P2 ratio, which may be due to the number of sperm used for sperm nuclear protein extraction, inefficiency in the commonly-used acid-urea gel electrophoresis method, or may indicate that differential regulation of protamine expression occurs amongst different men. Genetic abnormalities in the protamine gene or promoter leading to abnormality in protamine expression or P1:P2 ratio have indeed been widely reported in infertile men (Ravel *et al.*, 2007; Tuttelmann *et al.*, 2010; Jodar *et al.*, 2011). However, such genetic investigations have not been carried out in fertile men and therefore genetic mechanisms can not yet be related to the wide variation of P1:P2 ratio in previous studies.

Chapters 2 and 3 successfully demonstrated that cryopreserved sperm exhibit significantly reduced levels of P1 and P2. This represents a useful novel finding and suggests that the method developed herein can not only be useful in evaluating protamine content in cryopreserved sperm but also in sperm subjected to various other pre-treatments including sonication, incubation with Triton X-100 or PVP, treatment with HA medium, or even

following DGW. Further research may determine if these other treatments could also cause detrimental effects upon the total levels of protamine, and comparative studies could be performed to assess if any of these methods should be avoided in clinical practice. The present study also successfully demonstrated that an immunofluorescent assay can be potentially used as a clinical tool to provide information on the total levels of protamine in sperm and even allows visualisation of the localisation profile of protamine within the sperm head in either fresh or cryopreserved sperm. This is important because the expression and localisation profile of protamine within a sperm head would provide a useful indicator of DNA integrity and fertilisation ability of sperm.

Herein, I also used the acid-urea gel electrophoresis technique to evaluate P1, P2 and P1:P2 ratio in non-cryopreserved and cryopreserved sperm. Acid-urea gel electrophoresis is an established method to evaluate protamine levels in human sperm and a clear link between male infertility and protamine deficiency has been shown. The novel approach of this study was to use the same method to evaluate protamine levels and ratio in a mouse model. Interestingly, this method did not only show a significant reduction of protamine following cryopreservation, but also enabled efficient evaluation of P1:P2 ratio in mouse sperm. While this method represents a potential tool for protamine evaluation, it still requires an additional validation step to formally identify protamine identity in samples and this may incur more cost and time, and it therefore may not be useful for clinical practice as compared to the immunofluorescent assay, in which identity can be determined by simple peptide blocking rather than LC/MS.

The potential use of an immunofluorescent assay to evaluate protamine levels to determine DNA integrity of sperm was further justified when clear association between DNA fragmentation and protamine levels was demonstrated, especially when significant reduction of protamine was strongly associated with a significant increase in DNA fragmentation levels

in mouse sperm following cryopreservation. It would be also beneficial to use immunofluorescent screening to evaluate protamine levels and localisation profile in testicular sperm biopsied from patients to predict the fertilisation ability and DNA integrity of such sperm. Since current findings showed the clear effect of cryopreservation upon total levels of protamine in healthy mouse sperm, it is likely that this effect would also occur in human sperm and may be even greater in patients. This protamine immunofluorescent assay may also be useful as a routine screening assay in Sperm Banks and Research Institutes that produce genetically altered mouse lines and store frozen sperm such as the MRC Harwell to evaluate DNA integrity and fertilisation ability prior to utilisation for treatment or research.

The next step is clearly to investigate the application of the immunofluorescent assay in human sperm, particularly with regard to cryopreservation. It would be also useful to correlate protamine levels or localisation in human sperm with IVF or ICSI outcome. One future approach might be to perform functional studies such as ICSI or heterologous ICSI using cryopreserved sperm exhibiting protamine deficiency in comparison to non-cryopreserved sperm in order to evaluate whether fertilisation or early development is compromised. Collectively, these future studies may enable the extrapolation of this novel approach into clinical trials.

While Chapters 2 and 3 predominantly focused on investigating the effect of cryopreservation upon total levels of protamine, Chapter 4 expands upon the fundamental role of PLCζ, the sperm-specific oocyte activation factor in relation to male infertility and the importance of PLCζ screening for clinical application. There is an urgent need to expand our clinical understanding on how PLCζ deficiency relates to characterised states of male infertility. The experiments described in Chapter 4 represent the largest clinical screen of PLCζ in human sperm thus far, and identified several novel case studies involving infertility, PLCζ deficiency, and genetic abnormalities.

The specific underlying mechanism of PLCζ action is still not clearly understood and large scale clinical studies have yet to be carried out (Amdani *et al.*, 2013). However, several research outcomes from Chapter 4 further highlight the importance of this protein in determining the oocyte activation ability of a sperm and the success of fertilisation, especially following ICSI treatment (Kashir *et al.*, 2013b; Nomikos *et al.*, 2013a). ICSI failure is predominantly attributed to OAD (Vanden Meerschaut *et al.*, 2014), and a clear link between OAD and PLCζ deficiency in infertile human sperm has been widely reported (Yoon *et al.*, 2008; Heytens *et al.*, 2009; Taylor *et al.*, 2010; Kashir *et al.*, 2013b). Therefore, it is important to determine the total levels and localisation patterns of PLCζ clinically, as either a prognostic and diagnostic marker. Kashir *et al*. (2013b) first developed the PLCζ quantification assay using immunofluorescent technique in clinical samples and indicated that this immunofluorescent assay may limit its quantitative application as a clinical diagnostic indicator for oocyte activation, mainly due to the significant variability in the total levels of PLCζ, proportion of sperm exhibiting PLCζ and localisation patterning of PLCζ seen in 16 fertile controls and 5 infertile patients. Authors further indicated that while the total immunofluorescence levels of PLCζ do not represents as an efficient biomarker, however the PLCζ proportional analysis might potentially be a useful indicator to predict oocyte activation ability. Moreover, number of samples and sperm analysed may have limited the findings of Kashir *et al*. (2013b). Therefore, it is important to recruit a higher number of samples and to enhance our current understanding of PLCζ, and whether the PLCζ immunofluorescent assay could be potentially used as a clinical marker, when used in specific ways, especially when PLCζ quantification using Western blotting method is not necessarily useful at present due to lack of monoclonal antibody.

The first finding described in Chapter 4 was that a large group of fertile controls and infertile patients (n=30) showed wide variation in the total levels of PLCζ, the proportion of

sperm exhibiting PLCζ, and localisation patterning of PLCζ, similar to earlier reports by (Grasa *et al.*, 2008; Kashir *et al.*, 2013b). However, the novelty of the current dataset is that the variability in PLCζ led to the application of cluster analysis, which enabled the development of a novel PLCζ scoring and classification system. The PLCζ scoring system revealed four different classifications of PLCζ in both fertile controls and infertile patients. Clusters 1, 2 and 4 represented, high, intermediate and low classification of PLCζ respectively, in terms of total immunofluorescence level and proportional analysis. Cluster 3, represented a unique classification indicating a high proportion of sperm with PLCζ but with low levels. These crucial findings clearly suggested that there is a high possibility that the expression of PLCζ in sperm tends to lie within these defined ranges. The PLCζ scoring system developed based upon cluster analysis showed significant association with the success of IVF or ICSI. These data further suggest that the PLCζ scoring system can be potentially used as a diagnostic marker to predict the success of IVF or ICSI. In addition, linear regression models were generated correlating the total levels of PLCζ, and proportion of sperm exhibiting PLCζ, with fertilisation rates among 30 men. These regression models showed strong correlation which could also be used as a potential diagnostic and prognostic tool to predict fertilisation rates clinically. However, the low R^2 values obtained in these regression models may limit the clinical application of these tools, and further suggest that other unknown factors, besides PLCζ, may influence fertilisation rate following IVF or ICSI. It is important that future immunofluorescent work in the Coward Laboratory begins to use this PLCζ scoring system and regression models to further support and confirm the present findings. It will also be interesting for IVF units such as the OFU and ACU to begin using these PLCζ measures clinically to evaluate their potential worth.

Several infertile patients were identified as novel case studies in Chapter 4 and were specifically described due to their originality. For example, a vasectomy/vasectomy-reversal

patient who encountered ICSI failure exhibited significant deficiency in total levels of PLCζ. Interestingly, this patient fathered a child prior to vasectomy. This finding perhaps indicates that vasectomy/vasectomy-reversal may cause alteration in spermatogenesis leading to abnormalities in gene expression of sperm-specific proteins such as PLCζ, thus suppressing the expression of PLCζ. However, this hypothesis is speculative and it remains unknown as to whether vasectomy/vasectomy-reversal, or other surgical procedures, can actually lead to deficiency in PLCζ or any other sperm-specific protein. Moreover, the total levels of PLCζ in this particular patient prior to vasectomy remain unknown. Therefore, it is unwise to elaborate further on this finding, although several studies have reported the significant effect of vasectomy upon sperm structure, protamine content, DNA integrity, and gene expression in sperm (Thimon *et al.*, 2008; Sullivan *et al.*, 2011; de Vries *et al.*, 2013; Mahabadi *et al.*, 2013). In order to confirm this finding, future studies should aim to recruit a larger cohort of male patients undergoing surgical procedures such as vasectomy or vasectomy-reversal and perform routine PLCζ screening.

The link between PLCζ deficiency and OAD with ICSI failure is clearly evident, and previous studies have linked genetic mutation of PLCζ in exonic DNA to oocyte activation deficiency (Heytens *et al.*, 2009; Kashir *et al.*, 2012b). Interesting, a recent report by Pan *et al*. (2013) demonstrated mutation in the PLCζ promoter region of the Chinese Holstein bull, which significantly altered certain semen parameters. One specific case study patient described in Chapter 4, who encountered IVF and ICSI failure, was treated with AOA in his subsequent ICSI cycle leading to successful fertilisation. Importantly, this patient was diagnosed with PLCζ deficiency in the Coward Laboratory prior to AOA + ICSI treatment in the ACU, University of Dundee, Scotland. Therefore, it was evident that ICSI failure and OAD in this patient, who represented the first AOA patient in Scotland, was clearly linked to PLCζ deficiency. A particularly novel finding was that this patient exhibited an SNP in the

PLCζ promoter region. However, it remains unknown as to whether the PLCζ deficiency observed in this patient was associated with the SNP, and analysis continues to assess whether this is the case.

It is well established that male factor infertility can be commonly attributed to conditions such as azoospermia, oligozoospermia, asthenozoospermia, and teratozoospermia. Another condition affecting at least 0.1% of infertile cases, is known as globozoospermia (Dam *et al.*, 2007a). In this condition, sperm exhibit a round head devoid of an acrosome. Recent reports indicate that about 70% of globozoospermia cases are associated with genetic abnormalities in the DPY19L2 gene (Escoffier *et al.*, 2014). DPY19L2-deficient globozoospermic sperm exhibited complete loss or significantly reduced levels of PLCζ in human and mouse sperm. Since PLCζ was identified in the inner acrosomal membrane (IAM) and perinuclear theca (PT) of control human sperm, Escoffier *et al*. (2014) claimed that DPY19L2-deficient globozoospermic sperm may exhibit deficiency of PLCζ due to the lack of IAM and PT regions (Escoffier *et al.*, 2014).

Globozoospermic patients are frequently associated with OAD and PLCζ deficiency (Heytens *et al.*, 2009; Taylor *et al.*, 2010; Kashir *et al.*, 2012c). These patients are usually treated with AOA to rescue oocyte activation failure following ICSI (Heindryckx *et al.*, 2008; Taylor *et al.*, 2010). In the present study, significant deficiency in PLCζ levels was clearly demonstrated in a globozoospermic patient, with a low proportion of sperm showing PLCζ fluorescence in an abnormal localisation pattern in the anterior and mid-piece region of sperm, as described previously by Kashir *et al*. (2012c).

Genetic screening of our current globozoospermic patient revealed the same SNP in the PLCζ promoter as the AOA patient described earlier from Dundee. This was particularly interesting as PLCζ shares its bi-directional promoter region with CAPZA3, another sperm

protein involved in structural formation (Geyer *et al.*, 2009). One theory might be that the SNP detected in the globozoospermic patient described herein could have affected the expression of both PLCζ and CAPZA3 leading to OAD and a globozoospermic phenotype. However, this remains speculative at this point of time and represents another emerging research area in the Coward Laboratory.

Interestingly, two of the present case studies showed the same SNP in the PLCζ promoter. The common factor between these two cases is that sperm from these patients exhibited significantly reduced levels of PLCζ. However, the functional role of this SNP is still debatable as one patient exhibited normal sperm morphology whereas the other was globozoospermic. Moreover, this SNP has since been identified in a fertile control following genetic screening in the Coward Laboratory (data not shown). It is highly debatable as to whether such an SNP in the PLCζ promoter may be controlling the expression of both PLCζ and CAPZA3 in these patients. However, following verification and analysis using the genetic variation database from the National Centre of Biotechnology Information (NCBI), it became apparent that SNPs are common features of the PLCζ gene. Thus far, there have been 1338 SNPs identified in the PLCζ gene in a reference sample of 1000 fertile men. At present, nothing is known as to whether these SNPs are functional and the precise role of these SNPs in relation to PLCζ expression, male infertility or sperm morphology remains undetermined. There is also a possibility that these SNPs might be responsible for controlling the expression profile of PLCζ leading to the observed variability in total levels of PLCζ, and the differential pattern of Ca^{2+} oscillations in sperm from different men, especially in men categorised into the three MOAT groups, described by Vanden Meerschaut *et al*. (2013). Therefore, it is prudent for future studies to evaluate Ca^{2+} release profile in sperm exhibiting SNPs compared to controls. It will also be interesting to measure the expression and localisation pattern of PLCζ and CAPZA3 exhibiting SNPs compared to the wild type condition in mammalian

cells, such as HEK cells using a dual reporter construct. An additional quantitative immunofluorescent assay for CAPZA3, alongside immunofluorescent analysis, would also provide further evidence as to whether the SNPs may effect expression and localisation of both CAPZA3 and PLCζ in the sperm.

A recent theory suggested the involvement of an unknown oocyte factor in regulating PLCζ function inside the oocyte following gamete fusion (Yu *et al.*, 2012; Swann & Lai, 2013). This theory may further suggest that ICSI failure or OAD cannot necessarily be attributed to deficiency in PLCζ alone, but could also be related to deficiency in this as yet unidentified oocyte factor. In line with this oocyte factor theory, two patients were presented in Chapter 4 as special case studies. These patients encountered IVF and ICSI failure respectively. Intriguingly, sperm from these patients exhibited comparable levels of PLCζ to a fertile control man. Subsequently, both these patients were then subjected for treatment using donor oocytes in which one of the patient sperm was able to activate oocytes but not the other. There is a suggestion that previous oocyte activation failure may be attributed to deficiency in the unknown oocyte factor in the first of these patients. However, fertilisation failure in the second case appears to be related to a sperm factor deficiency which it was not apparent using our current diagnostic assays. It would be highly beneficial if future studies could include oocyte screening procedures such as MOAT, particle image velocimetry (PIV), PolScope and Ca^{2+} release assays to evaluate the quality, integrity and cytoplasmic maturation of oocytes in relation to oocyte activation, especially in the cases of OAD attributed to oocyte factors (eg. MOAT group 3 cases). Such screening would also allow the probability of selection and application of AOA treatment during ICSI treatment for the particular couples.

Male infertility can be attributed to advancing male age (Humm & Sakkas, 2013), given that abnormal sperm parameters and DNA fragmentation have been demonstrated in

sperm from aged men (Belloc *et al.*, 2014). In this thesis, the relationship between increasing male age and PLCζ were investigated for the first time. Findings clearly showed that there was no statistical association between total levels of PLCζ and proportion of sperm exhibiting PLCζ with increasing male age. Although data did not show association between age and total levels of PLCζ, it would be interesting to analyse the Ca^{2+} release profile of sperm from men of different age when injected into mouse oocytes.

Currently, the precise mechanisms underlying PLCζ expression still remain undetermined, although clinical and genetic links to PLCζ have been reported in published literature and in this thesis. Variability in the total levels of PLCζ and localisation patterning of PLCζ within a large cohort of males may be related to specific and as yet undetermined expression mechanisms. However, there are several possible mechanisms that may play a role in creating the observed variability in PLCζ expression. For example, it is known that sperm exhibit significant heterogeneity in ejaculated semen (Holt & Van Look, 2004). There is a possibility that the variability of PLCζ expression in sperm is simply related to such heterogeneity. The exact amount of PLCζ present in a single sperm is not known (Kashir *et al.*, 2014). However, it is estimated that mouse sperm contain 20-50fg of PLCζ (Cox *et al.*, 2002; Saunders *et al.*, 2002), whereas bull sperm contain 105-165fg (Cooney *et al.*, 2010). Thus, it is clear that levels of PLCζ may fall within a wide and as yet undefined range both across different species but also between different men, and perhaps within the same ejaculate.

There are six checkpoints which control gene expression in cells; 1) transcriptional control, 2) RNA processing control, 3) RNA transport and localisation control, 4) translation control, 5) mRNA degradation control and 6) protein activity control. The regulation of PLCζ gene expression at each of these checkpoints may influence the final amount of protein translated. Little is known of whether such mechanism influence expression of PLCζ

although Kashir *et al*. (2011b) suggested that mutations in the highly conserved region of PLCζ gene could affect their transcriptional/translational activity leading to instability and degradation of the PLCζ protein, thus compromising its functional role for oocyte activation (Kashir *et al*., 2011b). These mechanisms should therefore be investigated to assess whether any of these play a critical role in the expression of PLCζ in developing sperm. Moreover, with the discovery of SNPs in the PLCζ gene and promoter, there is a possibility that such SNPs may also exert influence over the expression of PLCζ and may contribute to the observed variability of PLCζ among men.

Finally, the observed variability in PLCζ expression could also be attributed to the polyclonal nature of the PLCζ antibody used for immunofluorescence staining. Thus far, all PLCζ immunofluorescence studies using our antibody here have shown consistency in the data obtained, and specificity has been proven with immunogenic peptide blocking experiments (Grasa *et al.*, 2008; Kashir *et al.*, 2013b). However, a similar experimental approach using a new monoclonal antibody would provide urgent confirmation of the immunofluorescent data. Whether the use of a monoclonal antibody may refute or confirm current data and existing published literature remains unknown. In order to generate a PLCζ monoclonal antibody, a purified form of PLCζ protein is required. Purified human PLCζ protein was successfully made by Yoon *et al*. (2012) and Nomikos *et al*. (2013a), and in the Coward Laboratory. Thus far, a monoclonal antibody has yet to be described in the literature. However, the Coward Laboratory is urgently seeking to address this shortfall by generating their own monoclonal antibody.

Mounting biochemical and clinical evidence strongly suggests the vital role of PLCζ as a clinical diagnostic and prognostic marker (Yoon *et al.*, 2008; Amdani *et al.*, 2013; Nomikos *et al.*, 2013a). A purified form of human PLCζ protein has significant potential to be utilised as a physiological therapeutic agent to rescue oocyte activation failure following

ICSI (Nomikos *et al.*, 2013a). However, recent controversial reports have refuted these widespread claims that PLCζ is the sole sperm factor and have presented another candidate protein, PAWP. Aarabi *et al*. (2012) first claimed that certain characteristics of PLCζ do not support its role as an oocyte activation factor. The authors claimed that PLCζ was a sperm-surface protein, and not cytosolic, as previously reported. They also reported that PLCζ diminishes during the acrosome reaction and does not diffuse into the oocyte following gamete fusion (Aarabi *et al.*, 2012).

The same group of researchers strongly believe that PAWP is the most relevant candidate for oocyte activation and presented further data in a series of subsequent papers. Immunofluorescent analysis of PAWP in human sperm showed localisation in the post-acrosomal sheath perinuclear theca, (PAS-PT), the most suitable region for an oocyte activation factor to reside. The PAS-PT develops and is assembled during the elongating spermatid stage, and this region is resistant to non-ionic detergent. This indicated that the oocyte activation factor does not only reside in this region, but should also be sustained in this region and incorporated into the oocyte following gamete fusion. This is not the case for PLCζ because non-ionic detergent extraction of human sperm led to the discoveries of PLCζ in the supernatant and not pellets. Moreover, total levels of PAWP were significantly correlated with fertilisation rates and embryonic arrest (Aarabi *et al.*, 2014a). A follow up study from the same research group carried out functional studies using ICSI with mouse and human sperm and performed Ca^{2+} release analysis. Injection of human and mouse sperm containing the PAS-PT region following non-ionic detergent extraction induced intracellular Ca^{2+} oscillations in both human and mouse oocytes (Aarabi *et al.*, 2014b). The authors also injected recombinant mouse PAWP protein and human PAWP cRNA into mouse and human oocytes respectively, which subsequently induced intracellular Ca^{2+} oscillations. Moreover,

these sperm-induced Ca^{2+} oscillations were successfully blocked with PAWP inhibitory peptides and an antibody against PAWP.

Another research group carried out crucial experiments in order to investigate the potential role of PAWP in oocyte activation (Nomikos *et al.*, 2014). These authors showed that NusA-tagged mouse PAWP protein was not able to hydrolyse PIP_2 *in vitro* and did not induce Ca^{2+} oscillations when injected into mouse oocytes. Moreover, microinjection of fluorescently-tagged PAWP cRNA failed to trigger Ca^{2+} release in mouse oocytes. Collectively, the work arising from these two research groups with regard to the potential relevance of PAWP is highly controversial, suggesting the urgent need for independent research to address the claims made by Aarabi *et al*. research group.

While research into PLCζ has predominantly focused upon investigating its essential role for oocyte activation, fertilisation and male infertility, it is also crucial to investigate whether sperm processing, manipulation and preservation during ART laboratory procedures could influence the levels of PLCζ and thus influence fertilisation rates. Kashir *et al*. (2011a) were the first to report the detrimental effect of cryopreservation upon total levels of PLCζ in human sperm. PLCζ was significantly reduced in sperm from fertile men following cryopreservation. Moreover, incubation of sperm in PBS at different time points has also led to significant reduction of PLCζ level (unpublished data from the Coward Laboratory). These earlier findings provided the foundation for investigations into other sperm manipulation techniques, such as sperm immobilisation by PVP during ICSI. In Section I of Chapter 5, the potential effect of sperm immobilisation via PVP upon PLCζ content was investigated in human sperm from cases recruited by the OFU. It was clearly demonstrated that PLCζ was significantly reduced in sperm from both fertile controls and infertile patients following PVP treatment, with sperm from patients showing higher vulnerability to PVP compared to controls. Changes in PLCζ localisation within the sperm head in controls and patients were

also observed. While Section I showed a clear effect of PVP upon PLCζ, it was important to note that such an effect was not observed in Section II of the same chapter in which the effect of PVP upon PLCζ was investigated in a larger number of sperm samples from donors. Prolonged exposure of donor sperm to PBS or PVP demonstrated a general trend of reduction in sperm viability, total levels of PLCζ, and the proportion of sperm exhibiting PLCζ. However, statistical analysis revealed that these changes were not significant and therefore that PVP did not influence viability or PLCζ. At present, the data from Sections I and II of Chapter 5 suggest that we can not firmly conclude whether PVP exhibits harmful effect upon PLCζ or not. The discrepancies between these two datasets could be attributed to the nature of the samples recruited in Section II, which were provided in a cryopreserved state, the low concentration of these samples, and the fact that the number of sperm analysed was not consistent among the donors. However, various studies have shown the deleterious effect of PVP upon sperm structure, DNA integrity, fertilisation rates and Ca^{2+} oscillations in oocytes injected with PVP-treated sperm (Strehler *et al.*, 1998; Yanagida *et al.*, 2001; Kato & Nagao, 2009; Kato & Nagao, 2012). Indeed, total fertilisation failure and abnormalities in embryonic development were observed following ICSI with PVP-treated sperm (Kato & Nagao, 2009). It remains unknown if these earlier data were associated with abnormal Ca^{2+} release as a result of PLCζ deficiency.

To our knowledge, the results described in this thesis are the first to report potential PLCζ deficiency in PVP-treated human sperm. It is prudent to expand such findings in terms of their potential clinical significance in case ICSI failure or OAD is somehow attributable to PVP treatment, at least in part. However, functional studies such as ICSI or heterologous ICSI using PVP-treated sperm to determine oocyte activation ability, Ca^{2+} release patterning, fertilisation rate, and potential effect upon embryonic development, first need to be carried out in the research laboratory prior to clinical testing. Several studies have also shown PVP

retention in oocytes following ICSI and that this may exert harmful effects upon the developing embryo leading to long-term problems and genetic defects in offspring (Jean *et al.*, 2001; Kato & Nagao, 2009).

Another important factor which needs to be taken into account is that it was recently shown that Ca^{2+} oscillation patterns induced by PLCζ within the oocyte following gamete fusion are correlated with the specific rhythm of cytoplasmic movements in activating oocytes, which can be monitored via particle image velocimetry (PIV) (Swann *et al.*, 2012). Cytoplasmic movements triggered following sperm entry are linked with re-organisation of mitochondria, endoplasmic reticulum, cortical granule exocytosis, and cytoskeleton. These parameters are also linked with cytoplasmic maturation and sperm-induced Ca^{2+} oscillation which is essential for embryonic development (Ajduk *et al.*, 2011). It follows that PLCζ deficiency could influence Ca^{2+} release pattern coinciding with rhythmical cytoplasmic movements within the activating oocyte, thus affecting embryo viability. Retained PVP within the oocyte may also be deleterious to the unknown oocyte factor involved in inducing PLCζ function. Moreover, PVP deposited into the oocyte during ICSI may detrimentally affect the organelles such as ER or cytoplasmic vesicles thus influencing the sperm-induced cytoplasmic movement compromising viability of the zygote. Collectively, such effects could lead to abnormalities in Ca^{2+} oscillation patterns, gene expression and embryonic development, and thus needs to be investigated in future studies.

HA is a physiological solution suggested to represent an alternative to PVP for sperm immobilization and sperm selection prior to ICSI (Parmegiani *et al.*, 2010). A preliminary study performed using sperm from a test control in the Coward Laboratory showed that sperm incubated with HA showed higher levels of PLCζ compared to PVP-treated sperm (unpublished data from the Coward Laboratory). It would be useful to perform comparative analysis between PVP-treated and HA-treated sperm compared to untreated sperm to

determine the significance of these treatments upon PLCζ in a larger number of sperm samples. This may lead to recommendations for improved clinical practice.

A number of exciting discoveries came to light during this thesis. Firstly, a novel immunofluorescent assay was developed for the quantification of P1 and P2 in mouse sperm. This could be extrapolated to human sperm and become a useful clinical tool. Secondly, cryopreservation was shown to clearly reduce protamine content in mouse sperm, indicating that similar studies are necessary in human sperm. Data confirmed that P1:P2 ratio does not change following cryopreservation in mouse sperm, however collective information from previous publications showed variation in P1:P2 ratio in fertile human sperm indicating that P1:P2 may not be a good marker to predict male infertility.

The work presented herein also provided significant new data to enhance our clinical understanding of PLCζ. Importantly, data confirmed the variable nature of PLCζ expression in human sperm, using a much larger cohort of patient than ever described before. Notably, a novel PLCζ scoring system and regression model correlating PLCζ and fertilisation rates with high potential for clinical utilisation were generated. Interestingly, several novel case studies were also identified, which linked PLCζ deficiency with specific clinical conditions, notably with vasectomy reversal. The identification of an SNP in the PLCζ promoter of two infertile patients was particularly exciting. Other data appear to suggest that PVP may have a detrimental affect on PLCζ, but this need to be confirmed in future, large scale studies.

Collectively, the data described herein enhances the potential of diagnostic and prognostic assays for the clinical evaluation of protamine and PLCζ, but also suggest numerous avenues of study for the future, both in terms of the mechanisms underlying PLCζ expression, but also the potential effect of laboratory or clinical procedures upon other crucial sperm proteins.

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Appendices

Genetic analysis and screening of the PLCζ gene promoter

1.1 Identification of the human PLCζ promoter

The online Ensembl database (http://www.ensembl.org) was first used to identify the human PLCζ promoter region. The DNA sequence encoding the promoter and exon 1 were then retrieved from Mutation Discovery software (www.mutationdiscovery.com).

1.2 Extraction of genomic DNA from patient saliva

Saliva samples from patient 7 and patient PM, recruited by the OFU and the Department of Reproductive Medicine at Ghent University (Belgium) respectively, were collected using FTA Cards (Whatman, UK) and an Oragene DNA collection kit (DNA Genotek, USA), respectively. Buccal cells present in the Oragene DNA collection kit were incubated for 1 hour at 50°C followed by addition of Oragene Purifier (DNA Genotek, USA). The cell suspension was then incubated on ice for 10 minutes and then centrifuged at 14,000g for 5 minutes and the pellet discarded. The supernatant was then mixed with 100% ethanol and incubated for 10 minutes at room temperature to precipitate DNA. DNA was then centrifuged at 14,000g for 2 minutes, the supernatant discarded, and the pellet re-suspended with 75% ethanol followed by centrifugation at 14,000g for 1 minute. The supernatant was then discarded and the pellet air-dried for 15 minutes to allow complete evaporation of ethanol. The air-dried pellet was finally re-suspended in nuclease-free water (Ambion Life Technologies, UK) and DNA concentration measured by spectrophotometry. Genomic DNA extraction from FTA Cards was carried out in accordance with the Manufacturer's protocol (Whatman, UK). Patient selection and ethical consent are described in **Section 4.2.1**.

1.3 Amplification of the PLCζ promoter by Polymerase Chain Reaction (PCR)

The genomic DNA encoding for the promoter-exon1 region of PLCζ was amplified from DNA extracted from patients 7 and PM using the High Fidelity PCR Master kit (Roche, Germany). The oligonucleotide sequences used are given in **Table 1.1**, and were previously designed in-house by Dr. Kevin Coward using Mutation Discovery Software (www.mutationdiscovery.com). PCR reactions were performed using a G-storm Thermal Cycler with an optimised cycle program shown in **Table 1.2**.

Table 1.1: Forward and reverse oligonucleotide sequences designed for the amplification of phospholipase C zeta (PLCζ) promoter-exon 1 region.

	Oligonucleotide sequence
Forward	GCCACCTTTCCCATCAAGGTGTT
Reverse	CAACACCATTGTGATTCCTGAAG

Table 1.2: PCR amplification program used to amplify the promoter/exon 1 of phospholipase C zeta (PLCζ).

Step	Temperature (°C)	Time (minutes/seconds)
Heated lid	110	
Denaturation	94	4.00
	Start cycle 1 (x10)	
Denaturation	94	0.10
Annealing	58	1.10
Elongation	72	2.00
	End cycle 1	
	Start cycle 2 (x20)	
Denaturation	94	0.10
Annealing	58	1.10
Elongation	72	2.00
	End cycle 2	
Elongation	72	7.00
Store	8	Infinity

1.4 Gel electrophoresis

Agarose gels were prepared by dissolving analytical agarose (Promega, UK) in 1X Tris-Acetate-EDTA (TAE) buffer (Fisher Scientific, UK) followed by the addition of ethidium bromide (0.625mg/ml, Fisher Scientific, UK). The molten gel was allowed to cool and set into a gel cast. DNA samples and a 50bp DNA ladder (Fermentas, UK) were mixed with loading dye (Fermentas, UK) and loaded into gel wells along with appropriate positive and negative controls. Finally, the gel was run at 120V for 1 hour prior to visualization using a GelDOC-IT TS imaging system (Ultra-Violet Products, USA).

1.5 PCR purification, DNA sequencing, and analysis

PCR products were purified using a QIAquick PCR purification kit (Qiagen, UK) following the Manufacturer's protocol. Amplicons were then sent for sequencing (Geneservice, Oxford). Comparative analysis was performed between PCR amplicons and the corresponding wild type human PLCζ region provided by Mutation Discovery Software (www.mutationdiscovery.com) and subjected to multiple sequence alignment using the ClustalW Multiple Sequence Alignment application (BioEdit Sequence Alignment Editor Software).

Appendix 2.0

Characterisation of genetic variation identified in the PLCζ promoter region using bioinformatics

Patient 7 and patient PM exhibited a single nucleotide substitution in the PLCζ promoter region. This substitution was investigated using a BLAST software provided by the University of California, Santa Cruz (UCSC) which revealed that this is was single nucleotide polymorphism (SNP: Reference: rs138662082) (**Figure 2.1**). When this data was directed to the 1000 genomes database, it was also noted that 1% of the sample population (n=1000) "healthy subject" exhibited this type of polymorphism.

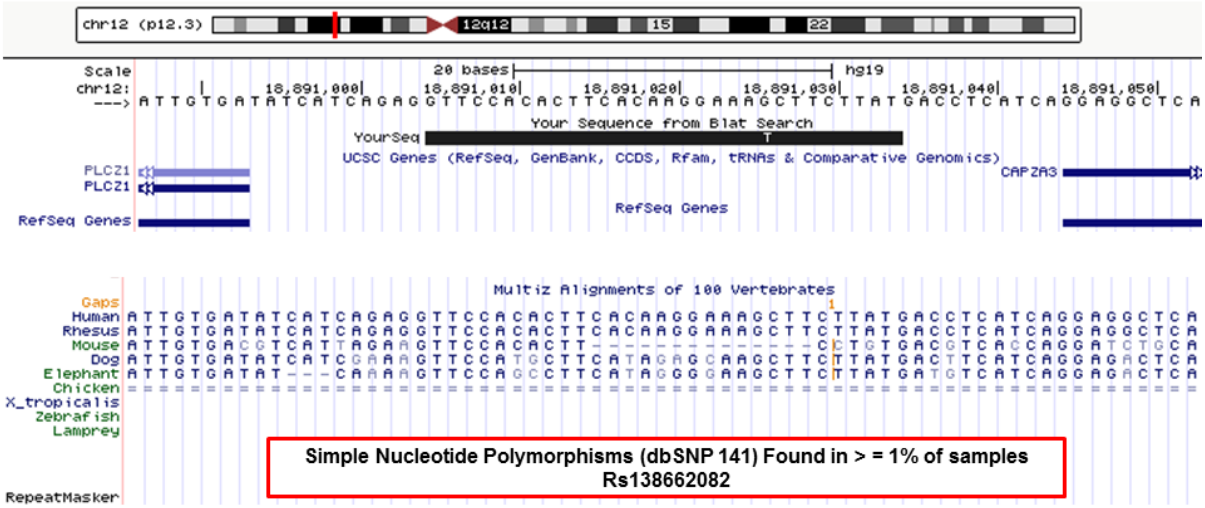

Figure 2.1: Screen capture of results retrieved from the University of California Santa Cruz (UCSC) database to determine the nature of the single base pair substitution identified in the PLCζ promoter region. Results indicate that the genetic variation identified was a single nucleotide polymorphism (SNP).

Appendix 3.0

List of publications and book chapters

Suseela Yelumalai, Junaid Kashir, Celine Jones, Hani Bagheri, Su Lin Oo, Laura McLaren, and Kevin Coward (2012). Clinician-induced (iatrogenic) damage incurred during human infertility treatment: Detrimental effects of sperm selection methods and cryopreservation upon the viability, DNA integrity, and function of human sperm. *Asian Pacific Journal of Reproduction*, 1 (1): 69-75.

Celine Jones, Hani Bagheri, Junaid Kashir, Laura McLaren, **Suseela Yelumalai**, and Kevin Coward (2012). The potential effect of clinician-induced (iatrogenic) damage incurred during fertility treatment upon gamete competence and embryonic viability. Chapter in Book entitled: Infertility: Genetic Factors, Treatment Risks and Benefits, Social and Psychological Consequences. Nova Science Publishers, *Elsevier*, New York, 77-94.

Junaid Kashir, **Suseela Yelumalai**, Celine Jones, and Kevin Coward (2012). Clinician-induced (iatrogenic) damage incurred during human fertility treatment: detrimental effects upon gamete and embryo viability and the potential for epigenetic risk. *Human Genetics & Embryology*, 2 (1): 1000e105.

Suseela Yelumalai, Celine Jones, and Kevin Coward (2013). Iatrogenic ('clinician-induced') damage incurred by human sperm during infertility treatment: postgraduate research and collaborative developments between the Universities of Malaya and Oxford. *Journal of University Malaya Medical Centre*, 16 (2): 1-6.

Sarah Francis, **Suseela Yelumalai**, Celine Jones, and Kevin Coward (2014). Aberrant protamine content in sperm and consequential implications for infertility treatment. *Human Fertility*, 17 (2): 80-89.

Suseela Yelumalai, Marc Yeste, Celine Jones, Siti Nornadhirah Amdani, Junaid Kashir, Ginny Mounce, Sarah Martins Da Silva, Christopher Barratt, Enda McVeigh, and Kevin Coward (2014). Total levels, localization patterns and proportions of sperm exhibiting phospholipase C Zeta (PLCζ) are significantly correlated with fertilization rates following intracytoplasmic sperm injection. *Fertility and Sterility*, (manuscript submitted).

Appendix 4.0

List of conferences and symposia

Suseela Yelumalai, Celine Jones, Su Lin Oo, Marisa Schubert, Junaid Kashir, and Kevin Coward. Potential iatrogenic damage during Assisted Reproductive Technology (ART) upon sperm protein expression, function & abundance. *Nuffield Department of Obstetrics and Gynaecology DPhil Day Symposium*, November 2012, Oxford, UK. (Oral presentation).

Su Lin Oo, **Suseela Yelumalai**, Celine Jones, Junaid Kashir, Marisa Schubert, and Kevin Coward. Levels and localization patterns of protamine 1 (P1) and protamine 2 (P2), essential proteins for sperm head condensation and DNA stability, in fresh and cryopreserved mouse sperm. *Developmental Biology Symposium*, December 2012, Oxford, UK. (Poster presentation).

Suseela Yelumalai, Celine Jones, Junaid Kashir, Su Lin Oo, Marisa Schubert, and Kevin Coward. Potential iatrogenic damage during Assisted Reproductive Technology (ART) upon sperm protein expression, function & abundance. "*Fertility 2013" Biennial Conference*, January 2013, Liverpool, UK. (Oral presentation).

Laura McLaren, **Suseela Yelumalai**, Celine Jones, Natalia Barkalina, and Kevin Coward. Effects of polyvinylpyrrolidone (PVP) upon the oocyte activation factor phospholipase C Zeta (PLCζ) in boar sperm. *Special Study Module Presentations, Medical Sciences Division*, April 2013, Oxford, UK. (Poster presentation).

Suseela Yelumalai, Celine Jones, Ginny Mounce, Enda McVeigh, Muhammad Fatum, and Kevin Coward. Levels of the oocyte activation factor, phospholipase C Zeta (PLCζ), in human sperm are reduced following incubation with polyvinylpyrrolidone (PVP). "*69th American Society of Reproductive Medicine (ASRM) Annual Meeting*, October 2013, Boston, Massachusetts, USA. (Oral presentation).

Suseela Yelumalai, Celine Jones, and Kevin Coward. The effect of cryopreservation upon total levels of protamine, a key sperm nuclear protein responsible for maintaining DNA integrity, in mouse sperm. *Nuffield Department of Obstetrics and Gynaecology Away Day*, March 2014, Oxford, UK. (Poster presentation, won prize for best poster).

Suseela Yelumalai, Celine Jones, and Kevin Coward. The detrimental effect of cryopreservation upon total levels of the sperm nuclear protein, protamine (P1 & P2) in mouse sperm: A novel method for direct protamine quantification using immunofluorescent techniques. *Nuffield Department of Obstetrics and Gynaecology DPhil Day*, November 2014, Oxford, UK. (Oral presentation).

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