CALCIUM SIGNALS AND THE ACTIVATION OF EMBRYO DEVELOPMENT IN MICE AND HUMANS

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MBChB, MSc

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Institute of Molecular and experimental Medicine

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Author's Declaration

I hereby declare that this Thesis has been composed by myself and is a record of work performed by myself under the title of "Calcium Signals And The Activation Of Embryo Development In Mice And Humans". This work was carried out under the supervision of Prof. Karl Swann. I conducted all the research at Karl's laboratory in the school of Medicine in Cardiff University, Cardiff, UK.

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ABSTRACT

During fertilisation the egg undergoes a series of remarkable activation events including a prolonged series of intracellular Ca^{2+} oscillations. The Ca^{2+} oscillations are necessary and sufficient to trigger egg activation and support pre-implantation embryo development. The mechanism of the signal trigger is well established and involves a sperm factor released into the egg upon gamete fusion. Phospholipase C zeta (PLCζ) appears to be the mammalian sperm factor responsible for triggering Ca^{2+} oscillations during fertilisation. Consistent with this idea it has been found that, some cases of male factor infertility have been attributed to either qualitative or quantitative deficiencies in PLCζ. Intra-cytoplasmic sperm injection (ICSI) has overcome many cases of male factor infertility. However, some ICSI cases still see partial or total fertilisation failure. To overcome such failures, artificial egg activation using various protocols has been introduced. Ionophores are the main agents used to trigger artificial egg activation in human eggs. The work in this thesis provides evidence that the human PLCζ protein is effective and more efficient in triggering egg activation and in supporting pre-implantation embryo development in mouse eggs than other activators. That was achieved via a direct comparison of various activation protocols in a mouse model of ICSI fertilization failure. Furthermore through the use of ICSI and Ca^{2+} measurements in mouse eggs my studies have provided further evidence that the number of Ca^{2+} transients during activation can influence development to the blastocyst stage. Moreover, my work also investigates what methods could be used for injecting PLCζ protein within a clinic setting. . Other work has attempted to investigate how Ca^{2+} oscillations during fertilisation may influence later embryo development by testing the use of probe of hydrogen peroxide in mouse eggs. Overall the studies provide an important step in the development of new therapies for treating patients having problems conceiving with ICSI treatment.

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Randa Sanusi

PUBLICATIONS

- **Sanusi R**, Yu Y, Nomikos M, Lai FA, Swann K. (2015)Rescue of failed oocyte activation after ICSI in a mouse model of male factor infertility by recombinant phospholipase Cζ. Mol Hum Reprod. 21.10; 783-91.

- Nomikos M, Sanders JR, Kashir J, **Sanusi R**, Buntwal L, Love D, Ashley P, Sanders D, Knaggs P, Bunkheila A, Swann K, Lai FA. (2015) Functional disparity between human PAWP and PLCζ in the generation of Ca2+ oscillations for oocyte activation. Mol Hum Reprod. 21.9; 702-10.

- Bradley J, Pope I, Masia F, **Sanusi R**, Langbein W, Swann K and Borri P**.** (2016) Quantitative imaging of lipids in live mouse oocytes and early embryos using CARS microscopy. Development. May 5 (Epub ahead of print).

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ACRONYMS AND ABBREVIATIONS

cDNA	Complementary DNA
PMCAs	Plasma Membrane Ca^{2+} ATPases
GFP	Green flurescent protein
PLCζ	Phospholipase C zeta
OGBD	Oregon Green Bapta Dextran
IVF	*In Vitro* Fertilization
ICSI	Intra-Cytoplasmic Sperm Injection
Ca^{2+}	Calcium ion
Sr^{2+}	Strontium chloride (SrCl)
CG	Cortical granule
PIP3	Phosphoinositide PI(3,4,5) trisphosphate
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PI 3	Phosphoinositide 3-kinases (class I)
ER	Endoplasmic reticulum
MPF	Maturation promoting factor
MAPK	Mitogen activated protein kinase
NEBD	Nuclear Envelope Break Down
IP3	Inositol (1,4,5) trisphosphate
IP3Rs	Inositol-(14,5) trisphosphate receptors
cRNA	Complementary RNA
SR	Sarcoplasmic reticulum
SOCE	Store-operated calcium entry
DAG	Diacyl glycerol
PKC	Protein kinase C
PMGCs	Primordial germ cells
NO	Nitric oxide
ABP	Androgen binding protein
FSH	Follicular stimulation hormone

LH	Lutinizing hormone
ZP	Zona pellucida
ROS	Reactive oxygen species
FRED	Fura-red
BAPTA	1,2-bis-(2-aminophenoxy)-*N,N,N′,N′*-tetraacetic acid
AM	Acetoxymethyl esters
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
kDa	Kilodalton
CICR	Calcium induced Calcium release
GTP	Guanosine tri-phosphate
SOAF	Sperm oocyte activating factor
PI-PLC	Phosphotidylinositol phospholipase C
PKC	Protein kinase C
hPLCζ	Human phospholipase C zeta
SH2	Src homology 2
PLCζWT	Wild type phospholipase C zeta
COSHH	Control of Substances Hazardous to Health
HFEA	Human Fertilization and Embryology Authority
JBIOS	Joint Biological Services
PMSG	Pregnant mare's serum gonadotropin
HCG	Human chorionic gonadotrophin
IP	Intra-peritoneal
M2	Gamete optimising medium from Sigma
HKSOM	Hepes buffered Potassium Simplex Optimised Media
ICCD	Intensified cooled CCD camera
PLCζ-luc	Phospholipase C zeta tagged with luciferase
cAMP	Cyclic adenosine monophosphate
PCR	Polymerase chain reaction
PtdIns3P	Phosphatidylinositol 3-phosphate
a.a.	Amino acid

PtdIns5P	Phosphatidylinositol 5-phosphate
a.u	Arbitrary unit
DTT	Dithiothreitol
RhD	Rhod dextran
PIV	Particle image velocimetry
rtPCR	Reverse transcriptase polymerase chain reaction
PM	Plasma membrane
IMM	Inner mitochondrial membrane
PTP	Permeability transition pore
PCD	Programmed cell death
ROS	Reactive oxygen species
CB	Cytochalasin-B
CD	Cytochalasin-D
cpYFP	Circular permuted yellow-fluorescent protein
TE	Trophoectoderm
ICM	Inner cell mass
PMGC	Premordial germ cell
ORF	Opening region frame
ART	Artificial reproductive technology

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Chapter 1

Introduction

1 Introduction

This introduction shall act as a general analysis of the areas that are directly related and relevant to the areas of my study. As the work that I have done was on mouse gametes, eggs and sperm I shall start with an overview of gametogenesis, in which an explanation of the creation of male and female gametes is given. Gametogenesis differs between male and female; oogenesis for females and spermatogenesis in males. The section to follow will be an overview of Ca^{2+} changes that occur during the process of fertilisation and how it affects embryo development, in addition to PLCζ effect. Mitochondrial production of reactive oxygen species in relation to Ca^{2+} changes during fertilisation and its effect on pre-implantation embryo development within an egg then shall be discussed.

1.1 Oogenesis

The primordial germ cells (PMGCs) start to develop in the genital ridge before birth in the female foetus, these PMGCs go through a series of mitotic divisions to give rise to what is known as oogonia (Monk and McLaren, 1981). At around mid-gestation in mouse these oogonia enter the first meiotic division and will reach the prophase of meiosis1 by the 5^{th} postnatal day. At this stage they will remain arrested in the ovarian cortex within the primordial follicle and from now on they will be referred to as primary oocytes. By the age of 3-5 weeks the mouse will only have around half of the original primary oocytes that it was born with as they will degenerate before reaching sexual maturation which is around 6 weeks of age (Sato et al., 2006, Mattson and Albertini, 1990). Although these primary oocytes are arrested they continue to grow in size from 12 and 30µm in mouse and human respectively to 15 and 85µm, and this growth is completed within 10-18 days (Breed and Moore, 1996). The growth in size is accompanied with other structural changes such as the gradual appearance of what is known as the zona pellucida (ZP). The zona pellucida is a 6.2µm layer containing around 3.5ng of protein surrounding all mammalian eggs. This coating of glycoproteins is synthesised and secreted by the growing primary oocyte (Bleil and Wassarman, 1980). It not only regulates the egg/sperm interaction but supports communication between the oocyte and the follicular cells during oogenesis (Wassarman, 1987). The ZP consists of 3 different of glycoproteins in mice while in humans and many other mammals it consists of 4 types, their proposed function is to act as receptors for the sperm. In mouse the ZP3 protein has been thought to be the 'primary receptor' for sperm binding and in

initiating the acrosome reaction which is essential if fertilisation is to take place (Nixon et al., 2007). This view has been recently challenged (Jin et al., 2011). The evidence put forward was that the sperm starts the acrosome reaction before contact with the ZP (Jin et al., 2011). The appearance is not the only change that occurs to the primary oocyte during its growth. This stage is characterised by the appearance of a large centrally located nucleus called the germinal vesicle (GV) and accumulation of molecules which are essential for embryogenesis. Moreover, it further expands in diameter to 80 and 120 µm in mouse and humans respectively (Breed and Moore, 1996). Every single arrested primary oocyte is contained within a primary follicle of many layers of follicular cells known as granulosa cells. The first layer of granulosa cells forms gap junctions with each other as well as with the primary oocyte. These GAP junctions are essential for regulation of meiotic maturation late in oogenesis. The observed growth rate of the oocyte is dependent upon the communication through the GAP junctions. It has been found that the absence of specific GAP junctions would lead to cessation of growth at the type 4 pre-antral stage at a diameter of around 50 µm (Carabatsos et al., 2000). Therefore it comes with no surprise that the number of granulosa cells in contact with the oocyte is a rate limiting step in this process (Brower and Schultz, 1982). This growth is responsible for the ability of the oocyte to pass into the later stages of meiosis triggered either by a hormonal signal *in-vivo* or release from the follicle in vitro. Primary oocytes are arrested as diploid cells that need to undergo meiosis in order to get fertilised.

Meiosis is a two stage process, including meiosis I and meiosis II, in order to produce a haploid gamete containing one set of chromosomes. GV breakdown is marked by the loss of its surrounding membrane it indicates the start of the first meiotic division. The chromosomes at this point will have arranged themselves on the spindle towards the periphery of the oocyte. The chromosomes equally segregate into each of the first two daughter cells, but the size of the daughter cells are dissimilar with one cell being about 30 times larger than its sister, this is due to unequal cytoplasmic distribution. Degeneration is the fate of the small cell which is called the first polar body and the larger cell is now known as the secondary oocyte that contains a full set of chromosomes. This again arrests at metaphase II of the second meiotic division (Figure 1.1).

1.2 Ovulation

One of the markers of puberty is the onset of menstrual cycle in humans which is accompanied by physiological changes of the hormonal levels. These changes will allow the arrested oocyte to enter the final stages of meiosis. At around 6 weeks of age mice are reproductively mature and their reproductive cycle is known as the estrous cycle and occurs every 4 days in laboratory conditions (Mattson and Albertini, 1990), each ovary will contain about 10^4 oocytes. The length of the cycle could be influenced and varied by the use of hormones and the mouse strain is one of many factors that affect the length of the estrous cycle in mice (Caligioni, 2009). Follicular stimulating hormone (FSH) secreted from the anterior pituitary gland will stimulate follicles that have reached a specific size and this will promote a further increase in size and will induce fluid collection (Caligioni, 2009). At this stage the follicle will be known as the graafian follicle which will start to migrate towards the periphery of the ovarian cortex where it will reach final stages of maturation after it will be released (McLean et al., 2012). The final phase of the cycle is where luitenising hormone (LH) secreted from the anterior pituitary gland surges and causes nuclear maturation before the oocyte's release. By the time that the follicle is ready for release the oocyte will have completed the first meiosis and contain a full set of chromosomes and will arrest at metaphase II of the second meiosis (Donahue and Stern, 1968). Certain canine species such as dog and fox are an exception where their oocytes are arrested as primary oocytes with intact GV's (Farstad, 2000). In mice about 8-12 eggs are released during ovulation and when ovulation is stimulated by injection of hormones this number doubles and a single mouse can give around 20-30 eggs in a single cycle.

1.3 Spermatogenesis

Spermatogenesis is a process of sperm formation and like oocytes they originate from the primordial germinal cells (PMGCs) (Donovan, 1998). Spermatozoa were identified as the primary component of the semen to cause fertilisation. In humans, during early development the PMGCs can be detected by the end of the 3rd week of gestation in the extra-embryonic mesoderm. Ameboid movement of the PMGCs allows their migration toward the genital ridge by the end of 6 weeks of gestation (Anderson et al., 2000).

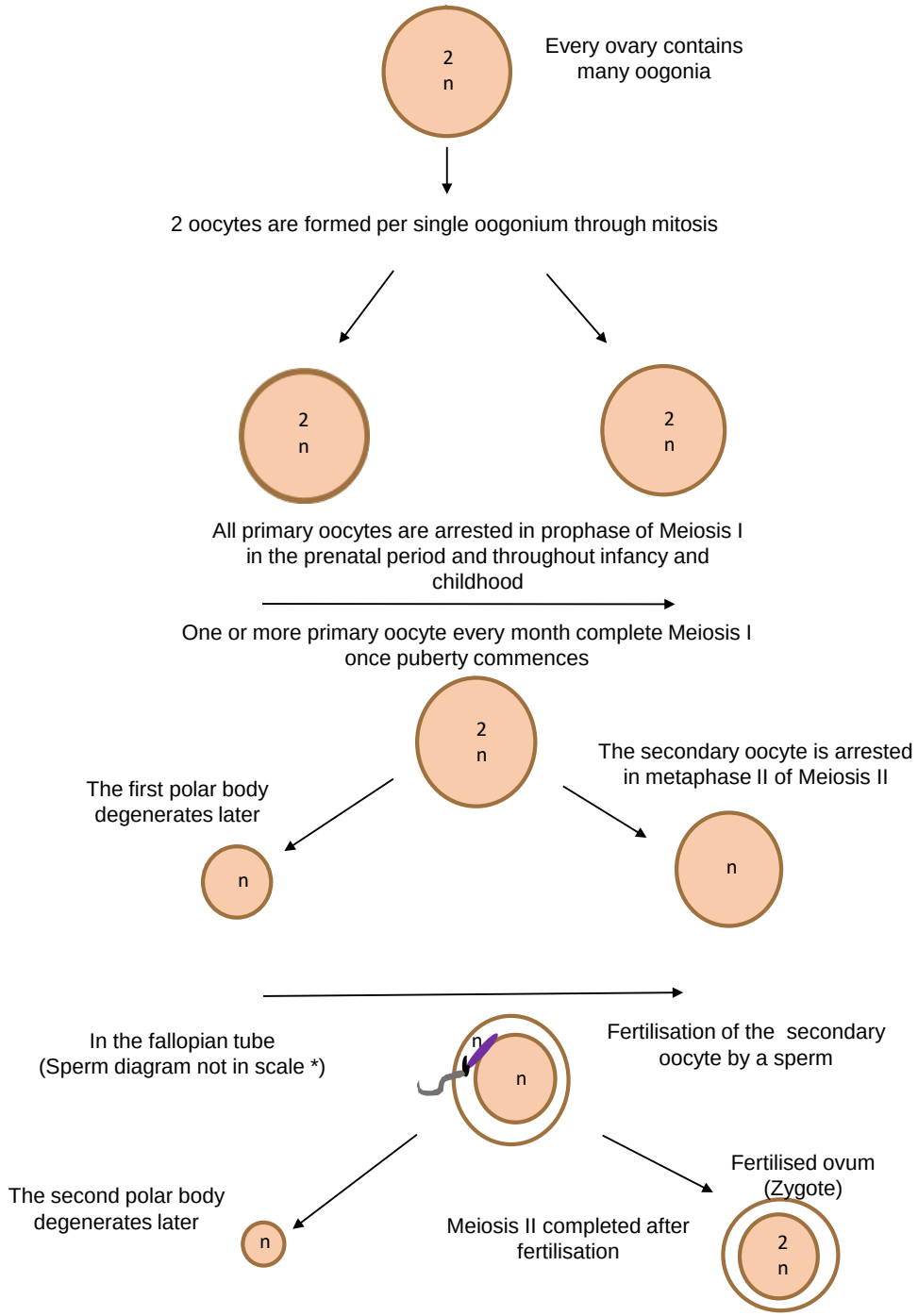

Figure 1.1 A schematic illustrating oogenesis and fertilisation.

The sperm diagram is not in scale as the egg diameter in reality is 20X greater than the diameter of the sperm head.

During their migration phase they undergo several rounds of multiplication and by this time the embryo is yet to gain sex differentiation (Stoop et al., 2005). A week later the embryo will have established its sex and formed its gonads. Normal spermatogenesis is maintained via the Y chromosome, yet a number of factors influence this process such as hormones, growth factors and vitamins (Skaletsky et al., 2003). Once the testis is formed the migrated PMGCs continue to proliferate into gonocytes until about 12 weeks of gestation where spermatogonia can be identified (Skaletsky et al., 2003). Spermatogonia are identified as the multi-potent stem cells of the germ line. The process of spermatogenesis is supported by two types of interstitial cells of the testis, sertoli and leydig cells. FSH and LH secreted from the pituitary gland regulate the function of both of these cells respectively.

The process of spermatogenesis starts by formation of diploid primary spermatocytes (46XY) via the mitotic division of spermatogonia (Stoop et al., 2005). The first meiotic division yields haploid cells (23X or 23Y) while 20X or 20Y in mice and these are known as secondary spermatocytes, and subsequently a second meiotic division occurs and spermatids are the result. Significant morphological changes occur during this process and continue during the postmeiotic period (Fig. 1.2). The rounded spermatid converts to a normal spermatozoon. At this stage the nucleus compacts, DNA condenses, mitochondria accumulates and form an axoneme, one of the cellular centrioles elongates to form the tail while the golgi apparatus form the acrosomal cap. These morphological changes take up to 6 weeks to finalise in human spermatogenesis (Heller and Clermont, 1963). The cycle of maturation from spermatogonium to spermatids takes around 65 days to complete (Dym, 1994). Cells from successive stages of the process arrange within the seminiferous tubules depending on their stage of maturation with the spermatids progressing towards the lumen. The process in itself is insufficient to produce sperm capable of fertilisation and the spermatids spend about 2 weeks to reach the cauda epididymis via peristaltic movements of the tubules. Once they get to the epididymis they attain their ability to fertilise via gaining motility. In mice the duration to produce sperm capable of fertilising an egg is almost half of that required in humans (Reijo et al., 1995). In humans 100 million sperm are produced daily and each ejaculate releases about 200 million sperm (Reijo et al., 1996). The sperm head is species specific, however, in all mammals the head contains highly condensed chromatin which is the DNA mass (Fig. 1.3) (Ward and Coffey, 1991).

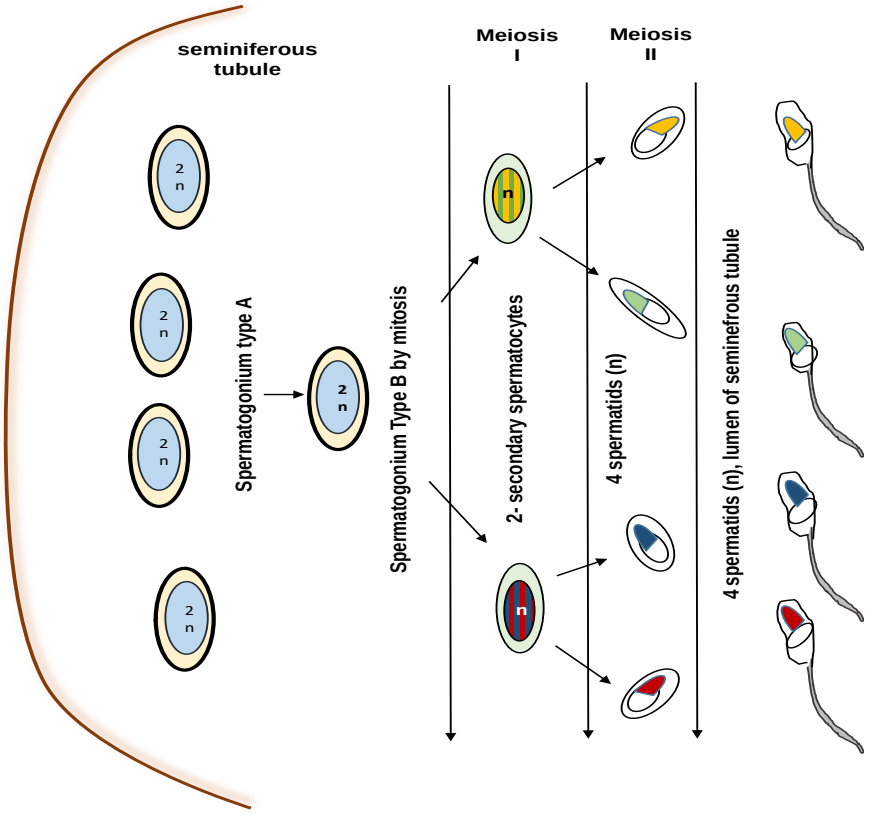

Figure 1.2 A schematic illustrating the maturation process during spermatogenesis.

As the process goes the maturation process is accompanied by the movement of the germ cells toward the lumen of the seminiferous tubule as the population of the germinal cells are located at the base of the tubule.

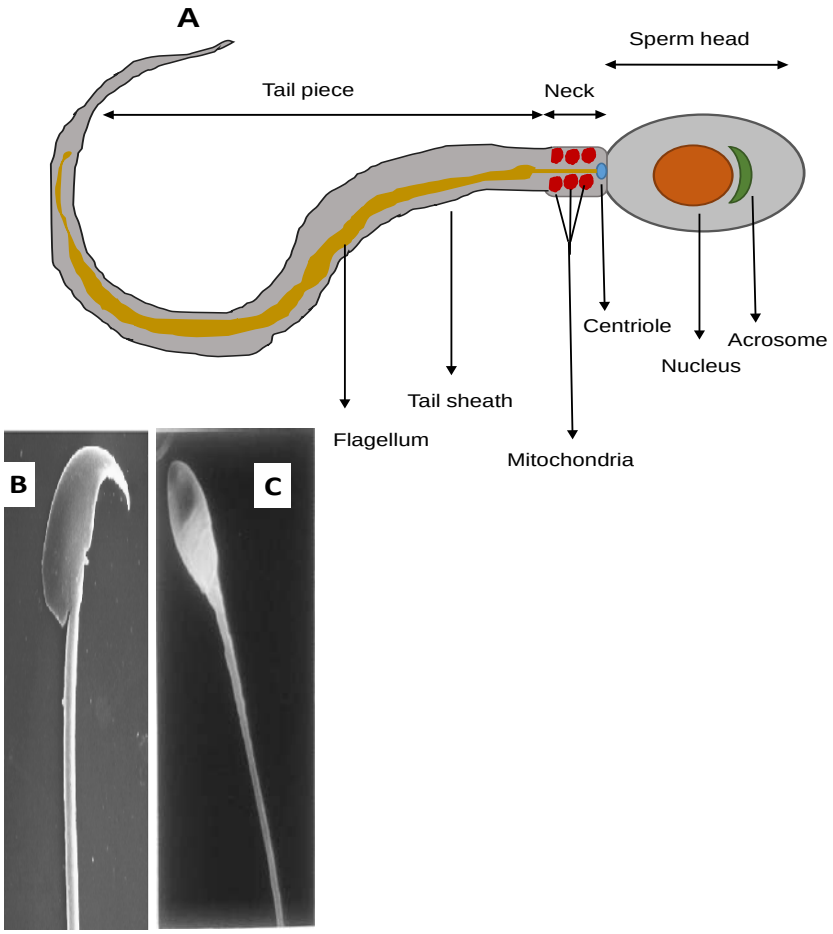

Figure 1.3 An illustration showing the main parts of the sperm.

A, a schematic representing the main parts of a sperm, the head containing the condensed haploid nucleus, mid-piece with mitochondria for energy production, the tail to propel the sperm through the aqueous medium in female genital tract.

B, Head of a mouse sperm. C, Human sperm with a morphologically different head from that of mouse.

1.4 Sperm capacitation

After ejaculation the sperm will have to acquire the ability to fertilise the egg although they are supposed to be mature and do appear to be normal, they are not able to do so until they undergo a process known as capacitation, this process occurs inside the female genital tract before it reaches the egg (Visconti et al., 1995). Chang in 1984 defined capacitation as a process where the sperm requires a certain length of time which could be in vivo or in vitro to bring about its final functional maturation (Chang, 1984). Capacitation as mentioned could be achieved using cauda epididymal and/or ejaculated sperm under a variety of different incubation conditions. *In vitro* capacitation refers to a method where the collected sperm is left in an optimal media within a humidified incubator set with an optimum temperature of 37^{o}C and CO_2 level of 5%. On the cellular level a number of changes do occur, levels of Ca^{2+} as well as cAMP are affected (Fraser, 1998), sperm swimming patterns change as does the membrane fluidity, protein phosphorylation activity and cholesterol efflux is seen as well (Breitbart, 2002). Phosphorylation of several proteins is an essential step in capacitation and in humans it has been shown that reactive oxygen species play a role in this process (Aitken et al., 1989, Aitken et al., 2012).

1.5 Acrosome reaction

Once the sperm completes the capacitation process it undergoes another unique process during its short life-span within the female genital tract. The changes that have occurred within the cell membrane of the spermatozoon during the capacitation process are decisive for the success of the acrosome reaction. This vital process is mainly characterised by exocytosis of the contents of the acrosome along with the release of vesicles formed by patches of the outer acrosomal plasma membrane. The process as a whole has been shown to have slow kinetics (t ½ =14minutes), the fastest step in this development is the cytosolic Ca^{2+} increase (t ½ =0.1 minutes). However, the acrosomal swelling seems to be the rate determining step as it consumes most of the time to be completed (t ½ =13 minutes). Basically the process involves rupture of the cell plasma membrane followed by the release of enzymes and the exposure of surface antigens (Fraser et al., 1995, Marín-Briggiler et al., 2003). It has long been thought that the site of the actual occurrence of the acrosomal reaction was the zona pellucida, the ZP3 glycoprotein has both sperm binding and acrosomal reaction triggering capacity. Therefore, it is generally

conceived that the reaction occurs as a consequence of the binding (Florman and Storey, 1982, Storey et al., 1984). Recently, in mice, it has been suggested that the cumulus cells surrounding the egg are vital in triggering this reaction. It has been found that eggs stripped from their cumulus cells were fertilised as an exception whereas the cumulus enclosed ones were fertilised as a rule (Jin et al., 2011)

1.6 Fertilisation and egg activation events

Amongst different species fertilisation occurs at different points of the cell cycle. However, MII is the most common stage where the mammalian eggs arrest and await for fertilisation to commence. Notable exceptions for this rule is the canine eggs that arrest at the prophase of first meiosis (Farstad, 2000), sea urchin eggs arrest after completion of meiosis and star fish eggs once gone through the second meiosis they can be fertilised without arresting (Whitaker, 1996).

1.6.1 Sperm-egg interaction

The first encounter between the sperm and the egg starts when the sperm comes in contact with the zona pellucida; this is a species specific event (Swenson and Dunbar, 1982). Once the sperm gets in contact with the surface of the ZP, receptors on the sperm head (Izumo 1) bind to the corresponding ligands found on the ZP. The identification of Izumo1 protein has been a breakthrough in the understanding of the fertilisation process as sperm lacking this receptor were unable to fertilise eggs (Inoue et al., 2005). It took about nine years for the egg receptor to be identified and was named “Juno”, again sperm failed to fuse to eggs lacking the receptor (Bianchi et al., 2014).

1.6.2 How does sperm activate an egg?

The mechanism by which a sperm activates an egg and fertilises it has gone through steps of evolution where theories have been proposed then refuted. Three models initially have been set as an explanation to how the sperm sets off egg activation. The conduit, contact and content models were the main proposed theories to explain the Ca^{2+} release mechanism and hence the start of fertilisation (Jaffe, 1991). I will provide some background of what is known about the Ca^{2+} increases before describing the sperm factor mechanism that currently has been widely accepted as the most likely mechanism for this process.

The first model states that the sperm acts as a channel to allow access for extracellular Ca^{2+} ions to pass into the egg cytoplasm via pores located on the

sperm cytoplasmic membrane (Fig 1.4 1). The initial rise in egg cytosolic Ca^{2+} caused by direct Ca^{2+} influx from the sperm was thought to induce a process known as Ca^{2+}induced Ca^{2+} release (CICR) (Jaffe, 1991). This particular model was supported by the findings of which showed that sperm egg fusion occurs prior to any start of Ca^{2+} increments (McCulloh and Chambers, 1992, Lawrence et al., 1997). However, further studies did not support this model as it was found that Ca^{2+} influx into the egg cytosol only had the ability to produce a single Ca^{2+} transient and did not lead to the signature Ca^{2+} changes in the form of oscillations recorded during fertilisation (Swann and Ozil, 1994).

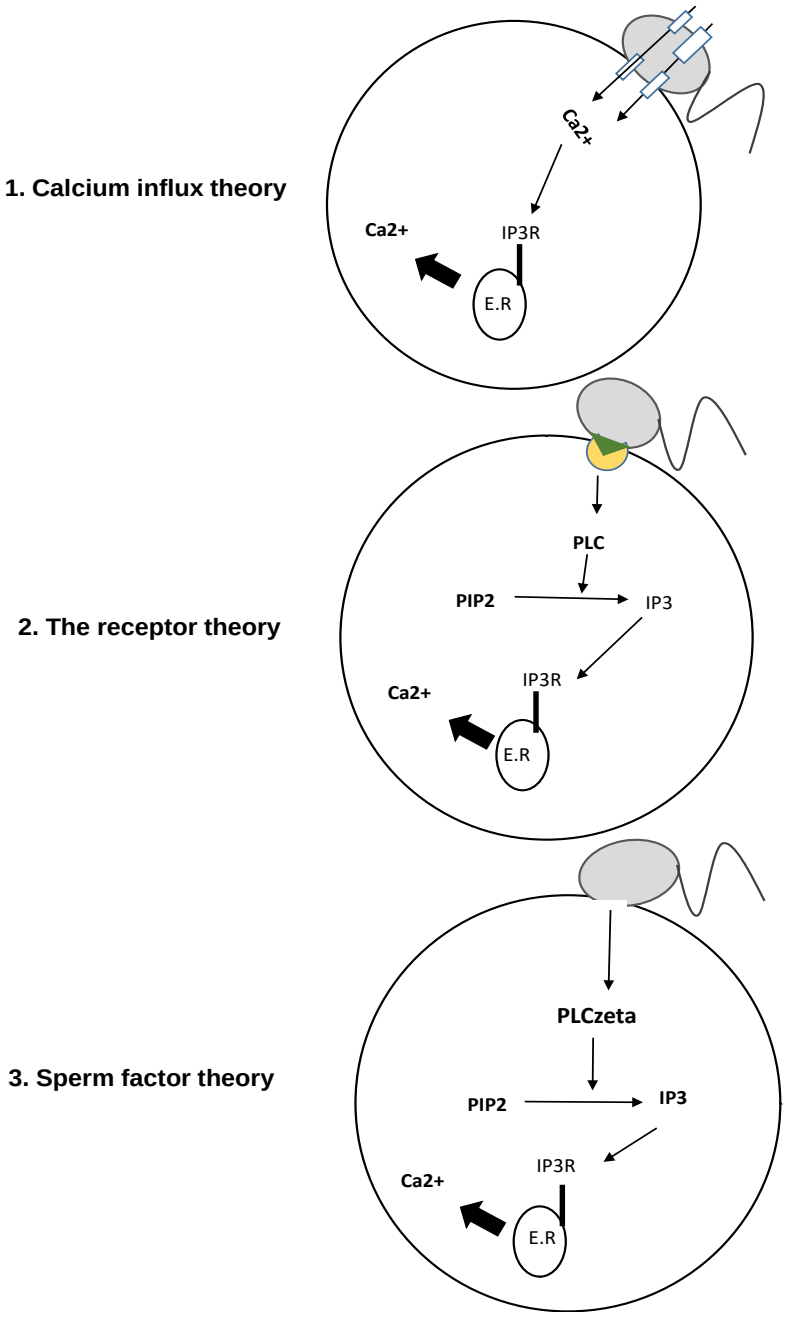

Figure 1.4 The 3 different theories explaining the calcium release in the egg during fertilisation.

All theories have a consensus on Ca^{2+} being the main trigger of egg activation. 1, sperm operating as a channel to allow Ca^{2+} into the egg, while 2, the receptor ligand theory activates a cascade of events after sperm-egg fusion starting with Ca^{2+} mobilisation. 3, the sperm factor theory allows an introduction of a phospholipase into the egg which triggers Ca^{2+} release from the ER.

The next theory is based on contact between sperm and egg through a receptor-ligand (Fig. 1.4 2). This theory is based on a signal transduction pathway where a sperm ligand interacts with a receptor on the cytoplasmic membrane of the egg (Foltz and Shilling, 1993). In spite of evidence supporting surface proteins facilitating sperm-egg binding there is no direct proof that this is linked to any intracellular signalling mechanism switching on (Runft and Jaffe, 2000) The receptor-ligand theory was suggested after studies revealed an increase in inositol 1,4,5-triphosphate ($InsP_3$) turnover during fertilisation in sea-urchin eggs (Ciapa and Whitaker, 1986). The receptor in action was thought to activate an egg derived PLC which in turn initiates phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis causing the release of $InsP_3$ acting as a Ca^{2+} releasing messenger. The proposed receptor on the egg was suggested to be coupled to a tyrosine kinase or a G protein signalling cascade that eventually will lead to Ca^{2+} increases within the egg cytosol. Again, this model was refuted on the basis that an antibodies developed to the Gq family of the G-proteins could not avert Ca^{2+} changes that occur during fertilisation in mouse eggs (Williams et al., 1998). However, there has been consensus upon the role of $InsP_3$ in regulating the Ca^{2+} oscillations since a number of studies showed that direct injection of $InsP_3$ into an egg caused a Ca^{2+} release in rabbits (Fissore and Robl, 1993), bovine (Fissore et al., 1995) and hamster eggs (Miyazaki, 1988). The main challenge to the receptor-ligand theory came in when a sperm was directly injected into the egg cytoplasm bypassing direct surface contact between both gametes, yet resulted in egg activation and embryo development with subsequent offspring delivery at term (Kimura and Yanagimachi, 1995).

This brings us to the sperm factor theory (Fig. 1.4 3), this theory proposes that once sperm egg fusion occurs, a soluble sperm factor passes through the pore formed into the egg cytosol and induce egg activation. This theory was supported when hamster sperm extracts were injected into eggs resulted in Ca^{2+} oscillations mimicking those seen during fertilisation (Swann, 1990). The sperm factor hypothesis explained what the conduit and receptor model came short of. Most importantly it could explain the possibility of fertilisation taking place without any sperm-egg surface interaction. Further support to this theory came from the demonstration of passage of fluorescent dextrans (Lawrence et al., 1997) and large molecular weight proteins (Jones et al., 1998b) between gametes once fusion occurs and this occurs before any recorded Ca^{2+} changes within the egg cytosol. Moreover, the gamete fusion itself precedes the Ca^{2+} change seen in sea urchin

eggs (McCulloh and Chambers, 1992). All of this data supported the notion that a soluble sperm factor is introduced into the egg cytosol after fusion.

Another interesting finding is that the proposed sperm factor functions across species resulting in Ca^{2+} changes and egg activation. For instance injecting porcine sperm extracts into cow, mouse (Wu et al., 1997) and hamster (Swann, 1990) eggs led to Ca^{2+} oscillations. Microinjection of sperm extracts from frogs, chickens (Dong et al., 2000) and fish (Coward et al., 2003) also has the ability to release Ca^{2+} in mouse eggs in a fashion similar to that seen during fertilisation. On the other hand when non-mammalian eggs such as nemertean eggs were injected with sperm extracts from mammals such as pigs, they responded with a pattern of Ca^{2+} of oscillations similar to those seen at fertilisation (Stricker et al., 2000). Collectively this data provided strong evidence that the sperm factor theory is highly conserved not only between mammalian species but across mammalian and non-mammalian species. Strikingly, this observation was established between the plant and animal kingdoms as well. That was when Li et al 2001 managed to show Ca^{2+} oscillations in mouse eggs resulting from injection of sperm extract of the plant *Brassica campestris* (Li et al., 2001).

During the early days of the sperm factor theory criticism was based around the amount of sperm extract injected into the eggs to induce Ca^{2+} oscillations, as it was predicted that more than what was equivalent to single sperm content was injected to result in a satisfactory level of response (Swann, 1990). It is worthwhile noting that the proposed sperm factor was a proteinaceous molecule and its extraction has been proved to be difficult hence the possibility of a significant loss of activity during this process. However, ICSI as a successful assisting reproductive technology has proved that a single sperm was sufficient to cause a physiological set of Ca^{2+} oscillations, egg activation followed by embryo development to term (Palermo et al., 1992, Tesarik and Sousa, 1994, Sato et al., 1999a).

1.7 ICSI and fertilisation failure

The improvements that have been seen in fertilisation rates due to IVF techniques has been predominantly seen in cases that have been diagnosed with tubal causes of infertility (Thonneau et al., 1991). Male causes of infertility had poorer rates of success (Tournaye, 2012). The introduction of the intracytoplasmic sperm injection (ICSI) technique has allowed cases with especially severe sperm factor infertility to

see high success rates in fertilisation and pregnancy (Van Steirteghem et al., 1993). In the early days of this technique the cause of activation was thought to be to the damaging of the membrane, as this was mimicking to some extent the sperm membrane changes that occur in the early egg sperm fusion (Sathananthan and Chen, 1986). It was even suggested that the low success rates when injecting a whole sperm was due to the active tail disrupting the egg membrane (Fishel et al., 1995). The breaking off the sperm tail in advance of injecting the sperm into the egg appears to be a vital step in the process, to allow for adequate sperm head de-condensation and subsequent pronuclear formation (Fishel et al., 1995). However, it was later suggested that a sperm cytosolic component was readily introduced into the egg cytoplasm by this method and many of the early biochemical changes that happen at fertilisation still do occur during ICSI as shown by Swann and Ozil 1994 (Swann and Ozil, 1994). The interaction between sperm and egg cytoplasmic membrane is not a pre-requisite for Ca^{2+} oscillations as proved by Ca^{2+} recordings following ICSI procedure and that when sperm extract were injected directly into an egg it caused Ca^{2+} oscillations and egg activation (Tesarik and Sousa, 1994, Homa and Swann, 1994)**.**

ICSI has the ability to overcome many causes of fertilisation failure, however it could not totally eliminate it. The additional problems include an exhaustive list of complexities (Swain and Pool, 2008). The prominent cause of failure in such cases is considered to be sperm defects. The sperm factor being defective is the leading basis of these causes (Heytens et al., 2008). Previous studies have shown a specific localisation of PLCζ in the sperm head of fertile men is highly likely to be in the perinuclear theca, while in cases of fertilisation failure following ICSI it has been shown to have an abnormal localisation pattern (Yoon et al., 2008, Kashir et al., 2010). In another study that assessed the ability of human sperm to cause Ca^{2+} oscillations in mouse eggs in order to identify sperm that can cause egg activation versus those that fail to activate eggs, the variability resulting suggests that human sperm are highly variable (Vanden Meerschaut et al., 2013a). It is worth mentioning that in this study only about 50% of the control sperm, that were considered to be from normal fertile male, that were injected into mouse eggs resulted into a frequency of Ca^{2+} oscillations that was considered to be of a normal pattern (Vanden Meerschaut et al., 2013a). Currently some fertility clinics are using artificial egg activators such as Ca^{2+} ionophores in attempting to overcome cases of activation failure and reasonably high success rates were reported (Taylor et al., 2010).

However, the current trend of using these chemicals could have potential risk on the developing embryo which could be mutagenic or even teratogenic (Nasr-Esfahani et al., 2010). In addition to the possible adverse effects, the Ca^{2+} changes using ionophores have no resemblance to the physiological pattern seen during fertilisation. The ionophores cause a single high rise of Ca^{2+} while the changes seen in fertilisation consist of a series of Ca^{2+} transients that last for a few hours (Swann and Lawrence, 1996). Therefore the pure PLCζ recombinant protein has the potential to stand as a viable alternative in treating these cases as it represents a physiological sperm factor that can induce egg activation (see later) (Nomikos et al., 2013).

1.8 Ca^{2+}; effects on Fertilisation and development

Ca^{2+} is considered as a universal intracellular messenger and acts as a stimulus for egg activation during fertilisation and is considered a vital component of initiating a fertilisation response in both animal and plant kingdoms (Whitaker, 2006, Li et al., 2007).

1.8.1 Ca^{2+} changes and fertilisation

Once the sperm enters the egg it elicits a Ca^{2+} change that can take many form depending on the species. In the majority of the mammalian eggs the change commences at the point of sperm entry and disseminates throughout the cytoplasm as a wave (Stricker, 1999). In marine worms sperm entry does not cause waveform Ca^{2+} changes, the Ca^{2+} change typically starts around the egg cortex where it peaks after in the nucleoplasm (Stephano and Gould, 1997). On the other hand non-mammalian eggs respond to sperm penetration by a single Ca^{2+} rise. Interestingly in some marine species the sperm is not responsible for the activation during fertilisation. In brine shrimp for instance, the ovulation process per se and the contact of eggs with water causes the Ca^{2+} change (Lindsay and Clark Jr, 1992).

Evidence shows that the source of Ca^{2+} change seems to come from internal stores, Ca^{2+} waves could be detected in sea urchin eggs when fertilised in media devoid of Ca^{2+} (Schmidt et al., 1982, Crossley et al., 1988). Mouse eggs managed to produce an initial Ca^{2+} transient even when the surrounding media had almost no Ca^{2+} (Jones et al., 1998a). Conceptually, the thought of Ca^{2+} being responsible for egg activation during fertilisation was postulated since the early 1930's from observation made during artificial activation of eggs. Ca^{2+} changes during fertilisation were

shown at fertilisation by the use of aequorin which is a protein that luminesces when it binds to Ca^{2+} in a variety of eggs (Cuthbertson and Cobbold, 1985, Whitaker, 2006). An alternative method also proved that egg activation is correlated with Ca^{2+} changes. In hamster eggs a series of hyperpolarisation was detected along with the series of Ca^{2+} changes during fertilisation. The explanation behind this association is that during a cytosolic Ca^{2+} rise activation of a potassium conductance leads to a membrane hyperpolarisation (Miyazaki and Igusa, 1981). A distinctive feature of the Ca^{2+} changes that occur during egg activation is that they take the form of a repetitive pattern named oscillations and they continue till around the time of pronuclei formation (Kline and Kline, 1992, Miyazaki i, 1993). The series of Ca^{2+} transients are not all uniform, in mouse eggs, the first Ca^{2+} transient typically takes longer to finish, but has a higher amplitude and on top of this transient smaller changes at the peak of this transient can be detected. The rest of the Ca^{2+} transients occur at a rate of about once every 10-15 minutes, have lower amplitude and last for a shorter time (Swann and Ozil, 1994).

1.8.2 Ca^{2+} changes and development

Physiological embryo development is totally dependent on the occurrence of Ca^{2+} oscillations; their necessity and sufficiency for the process of egg activation and promotion of further development have been proved so far (Kline and Kline, 1992). Egg activation is a process encompassing a number of phases including cortical granule exocytosis which prevents polyspermy, changes in synthesis of different proteins and exiting meiotic arrest (Ozil et al., 2005). If BAPTA-AM, which chelates Ca^{2+}, is added to the media, the total prevention of Ca^{2+} oscillations during fertilisation occurs and all events of egg activation are blocked (Kline and Kline, 1992). Furthermore, if Ca^{2+} oscillations are prematurely terminated, pronuclear formation is affected and progression to blastocyst stages will be influenced as well (Lawrence et al., 1998). Ca^{2+} spike number seems to play an essential role in the ability of the embryo to further develop. A maintained series of Ca^{2+} oscillations is to allow a sustained degradation of the maturation promoting factor (MPF) to allow release of the egg from its meiotic arrest (Ducibella et al., 2002). Otherwise it will remain arrested after extrusion of the second polar body in metaphase II. The need for a definite number of Ca^{2+} spikes is explained by the ability of the activated egg to form pronuclei after fertilisation via reducing the levels of mitogen activated protein kinase (MAPK) activity (Ducibella et al., 2002, Gonzalez-Garcia et al., 2014).

1.9 Post activation development

In mammals, once the pronuclei have developed a series of cleavages are initiated, and these cleavages are mitotic in nature and they are considered to happen at the slowest rate in the animal kingdom, typically occurring every 12-14 hours. Rotational cleavage is the mechanism for their occurrence, where one of the blastomere cells starts off by a meridonally cleavage the other divides equatorially. The blastomeres divisions do not synchronise, therefore, it explains the presence of an odd number of cells within the embryo at any given time point. About 17-20 hours post fertilisation the first cell division takes place yielding two identical daughter cells, about 24 hours later the second cleavage starts. By 60 hours post fertilisation the embryo reaches the 8 cell stage and blastocyst stage is formed by 84-96 hours (3.5-4 days) (Munne et al., 1995). A blastocyst develops due to the secretion of fluid into the morula, presence of Na^+ and K^+ ion pumps that cause a water flow by osmosis due to sodium movement into the extracellular space (Watson and Barcroft, 2001). Following the process of compaction the cells reorganise into two groups, the first is a layer towards the outside that forms gap junctions between themselves. This layer provides protection to the second group of cells that forms a mass in the inside. This specific arrangement influences cell differentiation as each group is exposed to different environmental factors. The early differentiation is then that the outer cells develop into the trophoectoderm (TE) giving rise to the placental structure and the inner cell mass (ICM) which will provide origin of fetal tissue.

Along with the cleavage activity, compaction and blastocyst formation the embryo moves towards the uterine cavity. Usually travelling embryos do not implant within the oviduct due to the presence of the zona-pellucida. Occasionally ectopic implantation occurs leading to a potentially life threatening haemorrhage when the oviduct can no longer accommodate the growing embryo and eventually ruptures. Once the expanding blastocyst reaches the uterine cavity two factors allow emergence of the embryo from its protective shell; the zona-pellucida. The expanding growth of the blastocyst by increasing the inside pressure from the fluid secreted along with its contractile activity and the action of Strypsin which has a trypsin like activity that aids in lysing the fibrillase material of the zona-pellucida(Watson and Barcroft, 2001). As soon as the embryo is released from its protective shell it will implant into the uterine epithelium which has transformed by a decidual reaction under hormonal influence to accept the embryo (Sandra, 2016).

1.10 Ca^{2+} effects on pre-implantation development.

Once the embryo has passed the first cell cycle, for the embryo to proceed with its development it requires Ca^{2+} oscillations at fertilisation to be with a specific pattern and intensity. Studies showed that when Ca^{2+} oscillations were manipulated in terms of frequency, amplitude and duration with the use of electrical field pulses (Ozil, 1998) they influenced the quality and quantity of developing embryos. The percentage of compacted embryos and further blastocysts where highly effected by the structure of Ca^{2+} oscillations as shown in rabbits (Ozil, 1990), cow embryos (Collas et al., 1993) and mouse embryos (Bos-Mikich et al., 1997). The duration of exposure to parthenogenetic activators such as Sr^{2+} affected the number of cells within the inner cell mass, increasing the duration of exposure stepwise from 2 to 4 to 6 caused a parallel increase in the cell number (Bos-Mikich et al., 1997). The early reports of cRNA encoding PLCζ injection into mouse eggs also revealed that it could support development up to blastocyst stage (Cox et al., 2002, Rogers et al., 2004). However, a study by Yu et al 2008 assessed the relationship between the level of protein expressed and the corresponding Ca^{2+} oscillations resulting along with developmental capacity. This study concluded that the higher the level of protein expression, the earlier the oscillation start with a higher frequency, an interesting finding was that the higher the frequency the earlier the oscillation would terminate (Yu et al., 2008). Another important finding was that there was a window of about 4 fold that PLCζ protein expression would translate into successful development to the blastocyst stage. The exact mechanism by which Ca^{2+} changes effect embryo development is yet to be known, but these data suggest that there may be a window of Ca^{2+} oscillations consistent with good development. This is consistent with observations in somatic cells where the frequency of repetitive Ca^{2+} transients influence gene expression more efficiently than the monophasic Ca^{2+} rise (Dolmetsch et al., 1998).

1.11 The quest to find the Sperm factor

Over the past years the search for the sperm factor was based on the evidence available that Ca^{2+} release within mammalian eggs is initiated following sperm egg fusion (Lawrence et al., 1997). The search needed to answer two important questions; 1 proposing and identification a potential molecule as a sperm factor and 2_ showing the proposed factor is capable of activating an egg physiologically.

Several proteins have come up over the years as sperm factor candidates. In the early days of the search for the factor, Ca^{2+} itself was proposed as the factor that initiates the activation process. The explanation was that Ca^{2+} was entering the egg cytosol from the surrounding environment. However, when Ca^{2+} was injected into the egg cytosol it failed to sustain a Ca^{2+} response in an oscillatory pattern (Igusa and Miyazaki, 1983). Moreover, the factor to be proposed had to be a protein as it has been proved with no doubt that the factor was a protein as its activity was shown to be abolished after protease treatment or heat treatment (Swann, 1990, Wu et al., 1997). A 33 kDa glucosamine-6-phosphate isomerase, oscillin, was one of the early candidates proposed and was extracted from hamster sperm extracts (Parrington et al., 1996). Successful recording of Ca^{2+} oscillation following injection of this protein that was present in semi-purified extracts was shown and the oscillations mimicked those seen during fertilisation. However, when the recombinant protein of oscillin was injected into mammalian eggs it failed to induce the response expected indicating that the protein identified was not the correct factor (Wolosker et al., 1998). Another candidate that attracted attention for a while was the truncated form of c-kit receptor known as tr-kit. About 30% of eggs injected with recombinant tr-kit or its complementary RNA managed to undergo the first mitotic division and reached the 2-cell stage (Sette et al., 1997). Criticism towards this proposed sperm factor was based on that there was no evidence that this factor could cause fertilisation induced Ca^{2+} oscillations and this was just one of the first points against this candidate. Another problem was that tr-kit was found to be located within the sperm midpiece which is usually not injected during ICSI and yet the egg still shows signs of activation. Therefore, the improper location was another element further eliminating this candidate as being the sperm factor. Moreover, the activation capability that was shown when eggs were injected with tr-kit was blocked with the injection of SH3 domain of PLCgamma. Injecting this construct into eggs that have undergone physiological fertilisation did not block the activation process, again indicating another mechanism of activation other than Ca^{2+} mediated one. All these combined data refuted the proposal of tr-kit as being the quested for sperm factor.

Another theory that did not survive for long was the proposal of nitric oxide (NO) as the sperm factor. This idea was based on the detection of NO induced Ca^{2+} increases in sea urchin eggs and both this release and the egg activation were blocked by NO-scavanger oxyhaemoglobin (Kuo et al., 2000). However, mouse and

ascidian egg studies failed to demonstrate any local or global NO changes associated with Ca^{2+} oscillations. Likewise, a nitric oxide synthase inhibitor could not block Ca^{2+} oscillations induced by fertilisation (Hyslop et al., 2001). Up to this point all candidates proposed failed to pass the definitive test to produce the distinct pattern of Ca^{2+} changes in the form of Ca^{2+} oscillations that are observed during fertilisation.

$InsP_3$ was also suggested as a sperm factor. A protein binding assay demonstrated that $InsP_3$ was present in a sufficient level within the sea urchin sperm that could activate an egg. The fact that the sea urchin sperm extracts starts to act when it's present inside the egg was perfectly consistent with the primary role of $InsP_3$ as a messenger (Iwasa et al., 1990). Disappointingly single injections of $InsP_3$ failed to cause Ca^{2+} oscillations, and the only effect seen was a single rise in cytosolic Ca^{2+} levels (Igusa and Miyazaki, 1983, Mehlmann and Kline, 1994). However, PLCs are known to directly cause an increase of intracellular $InsP_3$ synthesis, for that reason PLCs were thought to be the upstream cause of Ca^{2+} changes during fertilisation. A group demonstrated that when sperm extracts were added to a sea urchin homogenate, $InsP_3$ increments and Ca^{2+} changes were initiated and when the $InsP_3$ response in the homogenate was removed it led to a blocking of the sperm extract induced Ca^{2+} changes (Jones et al., 1998a). PLCs from now on were considered to be the obvious candidates for being the sperm factor and there were a number of PLCs that are known to be expressed in within the mammalian sperm such as PLC beta (β1), gamma (y1), gamma (y2), delta (d1) and delta 4 (d4) (Dupont et al., 1996, Mehlmann et al., 1998). With the fact that PLCs works via hydrolysing PIP_2 which produces $InsP_3$, it has been shown that when the PIP_2 is depleted from the homogenate the introduced sperm factor was incapable of initiating any Ca^{2+} release. But when the homogenate was rescued using external PIP_2 the Ca^{2+} oscillations started at once (Jones et al., 1998a). Moreover, supporting the PLC theory being the cause of initiating Ca^{2+} oscillations is was found that when a PIP_2-PLC inhibitor (U73122) was incubated with mouse eggs Ca^{2+} oscillations were blocked after sperm addition (Dupont et al., 1996). However, recombinant proteins of the former mentioned PLCs that were found to be expressed in mammalian sperm were unable to result in any Ca^{2+} changes that mimicked those seen during fertilisation or after injection of sperm or sperm extracts (Jones et al., 2000).

1.12 *The* sperm factor, a novel PLC

In 2002, Saunders et al identified a novel sperm specific PLC, later known by PLCζ (zeta), that caused Ca^{2+} oscillations mimicking those recorded during fertilisation, or after injection of sperm or sperm extracts into mouse eggs. Moreover, when the specific PLCζ was depleted from the sperm extract the Ca^{2+} oscillations were abolished (Saunders et al., 2002, Swann et al., 2004). Mounting evidence that the correct PLC isoform was the sperm factor, long quested for, came when the estimated amount of the novel PLC that was injected into a mouse egg and caused a response resembling that seen during fertilisation was comparable to the endogenous level of that detected in a single sperm. In both cases this found to be around 20-50 fg (Saunders et al., 2002). Furthermore, when cRNA encoding PLCζ was injected into mouse eggs it lead to activation and subsequent development to the blastocyst stage in rates similar to those seen during IVF procedures. Interestingly the newly identified PLC isoform was preserved across species, monkey and human PLCζ activated and supported development up the blastocyst stage in mouse eggs (Cox et al., 2002). Not only in mammalian species was the novel sperm factor preserved but when non-mammalian PLCζ from domestic chicken (gallus gallus) was injected into mammalian eggs (mouse) Ca^{2+} oscillations were detected as well (Coward et al., 2005).

1.13 PLCs characterisation

PLCs have been categorised into 6 different classes, delta (δ), beta (β), gamma (γ), …(η), epsilon (ε) and the isoform zeta (ζ) with each class having numerical subclasses making in total at least 13 different isoforms currently identified (Rhee, 2001, Hicks et al., 2008). Cell PIP_2 hydrolysis is the main mechanism of action for various PLCs, this step is critical in initiating egg activation as it is considered the signal transduction for this process (Fukami et al., 2010). A plethora of evidence shows that the phosphoinositide signalling pathway is stimulated once the Ca^{2+} oscillations commence during fertilisation which in turn leads to an increased production of inositol 1, 4, 5-triphosphate (IP_3) (Miyazaki i, 1993). The phosphoinositide-specific phospholipase C (PLC) enzymes when activated can generate IP_3 within the cell and in order to release IP_3 they catalyse the hydrolysis phosphatidylinositol 4, 5-bisphosphate (PIP_2). IP_3 thereafter binds to its receptor (IP_3R) on the endoplasmic reticulum where a conformational change opens the

channel causing an efflux of Ca^{2+} from the internal stores (Suh et al., 2008). It has been shown that the series of oscillation in cytosolic Ca^{2+} can be blocked by injecting monoclonal antibodies raised against IP_3R (Miyazaki et al., 1992).

Structurally PLC isoforms consist of various domains, some of which are highly conserved amongst different isoforms and some being characteristic for specific isoforms (Fukami et al., 2010). PLCs that have analogous domains share about 50% of the amino acid sequence in these regions, however there is no high similarity in structure between isozymes (Fukami et al., 2010). The X and Y domains are highly conserved domains in PLCs, functionally providing the catalytic site for PIP_2. The EF-hand motif is another example of a domain that is present in most PLCs as is the C2 domain (Rhee, 2001). Other domains include regulatory ones that can be in a different combination of forms in various PLCs. With the exception of PLC ζ, they all contain a PH domain (pleckestrin homology) which is thought to be used in a mechanism targeting membrane PIP_2 or PIP_3 (Harlan et al., 1994). Ca^{2+} is considered to be a universal activator across all PLCs, PLCζ and PLCδ have shown great sensitivity to Ca^{2+} levels in the physiological range indicating a possible regulatory mechanism for their function. More specifically it is thought that PLCδ is solely controlled by intracellular Ca^{2+} levels (Kim et al., 1999). In brief PLCδ activation is initiated by interaction between its PH domain and PIP_2, this will be followed by another interface mediated via its C2 region and the catalytic sites which will eventually lead to exposure of the active site resulting in the isoform activation and PIP_2 hydrolysis (Fukami et al., 2010). Protein-protein interaction is the usual mechanism allowing binding of the PLCs to plasma membranes. PLCδ is an exception as its PH domain binds with high specificity its target PIP_2. This interaction comprises a lipid-protein interaction (Nomikos et al., 2007). A specific activator for PLCζ is yet to be discovered, however, its extreme sensitivity to resting cytosolic Ca^{2+} may be sufficient to act as an activator (Saunders et al., 2002).

PLCs have been found to be expressed in different tissues. The brain is found to be the main expression site for PLCβ, while brain and lungs predominantly express PLCγ (Suh et al., 2008). Heart, lung and kidneys show high levels of PLCε expression (Song et al., 2001). On the other hand PLCδ is one of the most variable PLCs in terms of presence in different tissues as it can be found abundantly in brain, heart, lung, skeletal muscle and testes (Suh et al., 2008). PLCs that are considered to be more tissue specific include PLCη as it presents mainly in the nervous system

(Fukami et al., 2010). The novel PLCζ which at first was thought to be solely sperm specific has fulfilled criteria to be the sperm factor (Saunders et al., 2002). However, it is considered to be no longer a sperm specific phospholipase after recent evidence detection of PLCζ in the ovary of puffer fish (Coward et al., 2005).

1.14 PLC ζ, Structure and Function

Now that PLCζ has been recognised as the most promising sperm factor (Swann et al., 2006), research has moved on into the characterisation of this factor. The cRNA encoding this PLC isoform has proved to set off the Ca^{2+} changes similar to those triggered during fertilisation and can support embryo development up to blastocyst stage in all mammalian oocytes studied (Rogers et al., 2004). The reason behind the popularity of injecting cRNA encoding PLCζ is that the protein itself is a quite unstable *in vitro*, and so cRNA injection facilitated studying the characteristics of this PLC isoform. Amongst all PLCs identified PLCζ is considered to be the smallest protein (Fig. 1. 5) (Rhee, 2001). It consists of 608 amino acids to make the human form (Cox et al., 2002) which makes it smaller than the mouse form that comprises of 647 amino acids (Saunders et al., 2007). Although the human form is considered to be the smallest it has proved to be the most potent in inducing Ca^{2+} oscillations in mammalian eggs. The protein consists from 4 main domains.

1.14.1 EF hands

Phospholipase C zeta has four repeats of this motif located at the N terminus of the protein and they are arranged in a helix turn-helix motif (Miyazaki and Ito, 2006). Proteins containing EF hands upon Ca^{2+} binding are able to change their conformation. It is thought that their role in PLCs is to be responsible for the Ca^{2+} binding (Saunders et al., 2007). Initially plasma membrane PIP_2 was described as being the target for EF hands (Nomikos et al., 2005), however, recent reports have shown co-localisation of PLC zeta to intracellular vesicles within the egg cytoplasm (Yu et al., 2012), the precise nature of these vesicles are yet to be identified.

1.14.2 X-Y domain

This domain basically consists of three regions, the X-Y linker region and the catalytic domains X and Y. The linker region between X and Y is variable lengthwise amongst species, rhesus monkeys having the longest while the shortest is in humans (Saunders et al., 2007). The linker region resides between amino acid 308 and 385 with a total of 77 amino acids, it is highly variable in between PLC isoforms.

The nuclear localisation signal sequence is a unique structure and is located in this region. It is thought to be responsible for containment of PLCζ into the newly formed pronuclei during the fertilisation process in mice (Swann and Lawrence, 1996). The catalytic domains can be viewed as having a barrel shape, they are made up from alternating beta sheets and alpha helices (Saunders et al., 2007).

1.14.3 C2 domain

This particular domain is not exclusive to this protein it can be found in a variety of proteins such as protein kinase C with its different isoforms, phospholipase A and various isoforms of phospholipase C (Nomikos et al., 2011b). About 120 residues from this region are in almost all proteins with a C2 domain. This domain is structurally composed of four stranded β-sheets based in a sandwich of two each, their sequences show wide variation which might explain the range of lipids that they can interact with. Lipid interaction can take place by two different mechanisms, either in a Ca^{2+} dependent manner or a non-Ca^{2+} dependant interaction (Saunders et al., 2007). In PLCζ, the C2 domain is one of the vital components of the protein as versions with C2 truncation fail to induce Ca^{2+} oscillations in mouse eggs, despite preservation of its PLC activity in vitro. The C2 domain identifies specific phospoinositides to interact with, it can bind with both phosphatidylinositol 3 and 5 phosphate (Nomikos et al., 2005, Kouchi et al., 2005) while it fails to bind to phosphatidylserine (Saunders et al., 2007).

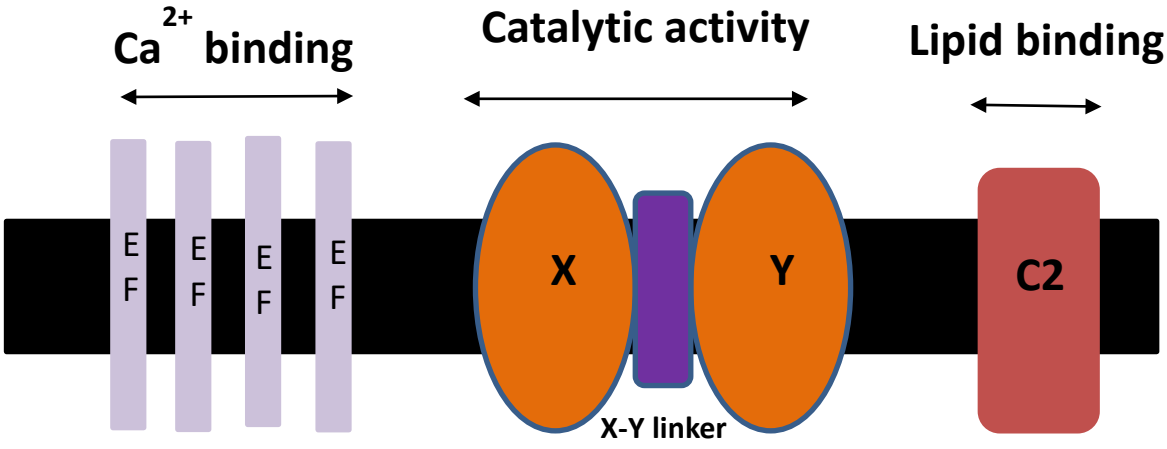

Figure 1.5 A schematic for the PLC protein with the various domain (647 aa))

1.15 PLCζ and its Ca^{2+} releasing ability

A unique character of PLCζ, is that it is the only PLC that can induce Ca^{2+} oscillations in eggs with injected concentration of only 1-100 femtograms. A domain which is present in all other PLCs and only absent from PLCζ is the PH domain. However, the simple lack of a PH domain does not explain the superior ability of PLCζ to cause Ca^{2+} oscillations. PLCζ has also been found to be the only PLC that is activated at Ca^{2+} levels as low as 100nM (Saunders et al., 2007). The lack of the PH domain in PLCζ is consistent across species and a similar domain structure is found in the chicken, mouse and monkey (Cox et al., 2002, Coward et al., 2005). It seems that the potency of PLCζ is not a feature conserved across species. The human version has been proved to have the strongest capability of inducing Ca^{2+} oscillation when injected in cRNA form (Cox et al., 2002). The exact cause for human PLCζ being the most powerful amongst others species is not precisely known yet, it is thought that X-Y linker region may contribute to this phenomenon as it appears to be the shortest of the PLCζ isoforms. One reason why it is potent is that the target for the human form which is the human eggs is considered to be the largest egg, therefore requiring a potent form of the egg activator. Alternatively it could be that it needs to be potent because of the sensitivity of the recipient egg to PLCζ. For example egg may differ in sensitivity to PLCζ since mouse eggs are more sensitive than hamster eggs in their response to sperm extracts (Parrington et al., 1996).

1.16 PLCζ and Male Infertility

Since it has been postulated that PLCζ is the sperm factor that causes activation of the egg and its release from its meiotic arrest during fertilisation, it is logical to relate some causes of male infertility problems to lack or deficiency of this factor.

1.16.1 Possible genetic defects in PLCζ

It has been established that some cases treated for infertility problems that the male partner had a lack of PLCζ in their sperm and the sperm failed in activating the eggs (Yoon et al., 2008). It was found in a single case with repeated history of failure of fertilisation during IVF/ICSI procedures that the sperm was globozoospermic (round head) and these sperms lack the acrosome in their structure. When a genomic analysis for the DNA of the PLCζ gene was done for this case, interestingly a defect in the opening frame of the DNA sequence was identified (Heytens et al., 2009). The defect identified was a change in the base sequence at the position 1193 of the

ORF, this was a change of the adenosine (A) by a cystosine (c) and this was predicted to be a mutation in the Y catalytic domain which could explain the infertility cause (Heytens et al., 2009). This point mutation has been confirmed as the reason of failure in causing the physiological series of Ca^{2+}oscillations. This was shown in a study by Heytens et al, involving two separate injections of mouse eggs with cRNA encoding hPLCζ one as the wild type ($hPLC\zeta^{WT}$) while the other had the point mutation ($hPLC\zeta^{H398P}$). The response to these injections was clustered into two groups, the first group demonstrated a series of Ca^{2+} oscillations mimicking those seen during fertilisation and those belonged to the eggs injected with the wild type hPLCζ. The second group either revealed a very minimal response where most eggs did not show even a single Ca^{2+} spike (Heytens et al., 2009). The results of this study confirmed that the mutation identified obviously affects the activity of the protein *in vivo*.

1.16.2 PLCζ, its Possible Role in Treatment of male Infertility.

In cases of male factor infertility due to defects in PLCζ, the activating signalling system which is Ca^{2+} is the missing link to release the egg from its meiotic arrest and the initiate development. Therefore, it would seem reasonable to assume that if the egg received a dose of PLCζ during ART then the eggs would more likely be able to activate. Moreover, other applications would include the use of PLCζ as a parthenogenetic activator in nuclear transfer protocols in generating embryonic stem cells. The cRNA encoding PLCζ, when injected into human eggs that have failed in an assisted reproductive technique, demonstrated a prolonged series of Ca^{2+} oscillations and development up to blastocyst stage (Rogers et al., 2004). PLCζ in its unique capability of causing a physiological Ca^{2+} response was considered to be superior to other agents used in activation protocols as some of these agents can only induce a single Ca^{2+} rise such as Ca^{2+} ionophores (Nasr-Esfahani et al., 2010). However the use of a genetic based material in clinical settings is not likely to be accepted, moreover, the amount of protein to be expressed is unpredictable after the injection of the cRNA, and this could cause adverse effects (Ozil et al., 2006).

1.17 Embryo Quality and Developmental Capacity

The main aim of any assisted reproductive technique is to transfer blastocysts that would potentially implant into the recipient uterus, the current guidelines is to transfer a single blastocyst or maximum two at each IVF cycle to reduce the risk of multiple gestations (Min et al., 2006). The *in-vitro* culturing environment cannot

support developing of an embryo beyond the blastocyst stage as at this point they need to implant. Embryo implantation rates when transferred at the blastocyst stage stage are around 30% to 50% (Gardner et al., 1998). It is the quality of the blastocyst transferred that determines the success rate of implantation and further development, therefore a number of parameters have been set to determine which embryos that are more likely to implant (Bavister, 1995, Van Soom et al., 1997).

1.17.1 Criteria for Embryo Quality Assessment.

An important topic since the introduction of IVF techniques is embryo selection. Initially embryo morphology was the most obvious criterion to base the selection upon. However, selecting the most competent implanting embryo based on its external features has limited predictability. Invasive procedures where embryos undergo a cell removal at a specific pre-implantation stage was found to negatively impact further development (Kirkegaard et al., 2012). What could be gained through analysis of cell markers from a single blastomere would be counterbalanced by the effect of its removal. Due to pitfalls of the invasive procedures, non-invasive methods seem to have wider acceptability when it comes to identifying best embryo for transfer in IVF clinics. However, it has to be noted that the increase in the number of successful pregnancies post IVF procedures has been attributed to the vast improvement seen within IVF protocols and not to the selection criteria introduced (Cohen et al., 2012).

1.17.2 Embryo Morphology, is it a valid tool for selection?

To select an embryo based on morphology during a single time point is subject to variability, as varying the time points that the embryo is examined at could reveal different findings (Montag et al., 2011). In an ESHRE meeting in 2011 the only consensus in regard to morphological based criteria that would have a valid prognostic marker was the presence of the smooth endoplasmic reticulum, as it would indicate an unfavourable pregnancy outcome (Otsuki et al., 2004). Another non invasive approach is to extend the period that the embryo are cultured. As most of the developmental blocks occur in the early stages, culturing to the blastocyst stage has been widely used since the enhanced medias and culturing protocols were introduced (Gardner et al., 1998, Summers et al., 2000). However, again this brings back the question over which blastocyst is more likely to implant if returned? Blastocysts have a more diverse appearance and several scoring systems exist (Dokras et al., 1993, Gardner and Lane, 2003, Gardner et al., 2015). Moreover,

recent studies revealed that the day 2/3 embryo produced from IVF were returned into the uterus were far more efficient in producing clinical pregnancies than when blastocyst stage was transferred (Cruz et al., 2012).

1.17.3 Embryo Metabolic Activity.

When extended culture media for blastocysts were designed it brought into light that the embryo has different needs at different times, which if not fulfilled would affect development and later implantation capacity (Gardner et al., 2001). Embryo metabolic activity was based on criteria such as oxygen consumption, nutrient uptake and waste production and embryo viability assessment built on this ground proved to be useful (Gardner et al., 1994). A number of target molecules were observed to have a changing pattern over development, glucose, pyruvate and amino acids were amongst molecules extensively studied from the media surrounding embryos. For instance glucose consumption positively correlates with progress in development and it may allow prediction of an embryo sex during the compaction phase (Gardner et al., 2011). Amino acids profiling on the other hand aided in identifying embryos with aneuploidy (Picton et al., 2010). The metabolomics field has attracted wide interest, but the technical difficulties and the need for high sample throughput questions the applicability of this method in routine clinical use (Gardner et al., 2015).

1.17.4 Time-Lapse Imaging, an influential tool for embryo selection.

Keeping the embryos from being disturbed by exposure to external environment during morphological evaluation was the main driver behind producing a technology that would efficiently reduce this concern. A further reason is that embryo development is a kinetically active process and the static method of monitoring once per day did not reflect truly the developmental process. In fact several important aspects to the morphology of the embryo could be missed. So the answer was constructing a device that could record changes without the need to be removed from the culturing environment. A number of experimental approaches were suggested (Lemmen et al., 2008), however, it was not until commercial time-lapse systems were introduced to the market for their full potentiality to be discovered (Montag et al., 2013). Time lapse imaging per se has not caused any decline in developmental rates in embryos (Kirkegaard et al., 2012), in fact it has been claimed that successful pregnancy rates have seen a rise of about 20% since its introduction (Meseguer et al., 2012).

Time lapse cinematography is a method used to follow embryos post fertilisation. One of the early parameters that showed the ability to predict superior quality embryos for implantation was the cell cycle timings, also known as the time taken for cleavages to finalise (Bavister, 1995, Van Soom et al., 1997). In cow embryos the fastest cleaving embryos had the highest implantation rates (Grisart et al., 1994), furthermore, it seems that when cleavage cycles synchronise during the first few cell cycles it would predict a favourable developmental course (Cruz et al., 2012, Wong, 2010). Differences do exist between embryos cultured *in-vivo* and *in-vitro* environments. For instance blastocyst hatching mechanism *in-vitro* occurs via focal lyses by the trophoectoderm projections while *in-vivo* blastocysts escape the zona by global dissolution (Gonzales and Bavister, 1995). However, blastocyst hatching criteria such as timings and dynamics is still a valid tool for assessing embryo quality.

1.17.5 Apoptosis and cellular quantification

Cytoplasmic fragmentation, also known as apoptosis, is a phenomenon observed in both superior and low quality embryos. However, the level of fragmentation does differ immensely, in excellent quality embryos it is thought that a minimal degree of fragmentation is required in aid of defective cells removal and to control cell number and growth (Hardy et al., 2003). On the other hand, where extensive fragmentation is present it would be associated with a reduction in the number of cells within the blastocysts, a decrease in the implantation rates and it has been recognised as a cause of a well-known phenomenon, embryonic arrest (Jurisicova et al., 1996, Hardy et al., 2003). The negative effect of fragmentation on the developing embryo could be explained in two mechanisms, first the dying cells could release toxic substances that could be detrimental to the healthy neighbours. Secondly, the cell death leads to a reduction in the amount of cytoplasmic material which means a potential loss of probably critical cellular components (Alikani et al., 1999). A plethora of quantification protocols for both embryonic cell types, the trophoectoderm and inner cell mass (TE and ICM) which highlights the importance of cell number distribution between these two cell types(Adjaye et al., 2005, Koo et al., 2002). A minimum number is needed for each cell type to allow normal fetal development (Hardy and Handyside, 1996) For instance, an increase in the ICM number over the TE has been linked to large offspring syndrome (Leese, 1998).

1.18 Embryonic Developmental Block.

The period following the event of fertilisation is considered to be a silent phase in terms of transcription and early development is driven via maternal mRNAs and proteins which are progressively degraded following fertilisation. Egg maturation is the onset for the degradation process and by the 2-cell stage 90% of maternal mRNA is removed, however, some of maternal protein is still synthesised up to 8-cell stage (Paynton et al., 1988, Zheng et al., 2005). The switch of the embryonic genome is activated at different times in different species, in humans it occurs at the 4-8 cell stage (Braude et al., 1988), while in mice it ensues once the 2-cell embryo (Flach et al., 1982). Therefore the developmental block is considered to be species specific event and is correlated to the activation/switch on of embryonic genome (Minami et al., 2007). Moreover, a number of factors seem to contribute to the developmental block; the strain of mice used and the culture conditions including medium components (Chatot et al., 1989).

In the early efforts of embryo culture, the 8-cell embryos were cultured up to the blastocyst stage in a simple defined media (Whitten, 1956). The effect of media on embryo development was soon recognised and as protocols for media improvement took place the culture of early embryos was seen as well. CZB is one of the first media that embryos from different mice strains were successfully cultured to the blastocyst stage (Chatot et al., 1989). Other media quickly followed; a simplex optimised media (SOM) along with the version containing potassium (K^+) supplement (KSOM) (Lawitts and Biggers, 1991, Lawitts and Biggers, 1993). Other factors contributed to the success of the new mediums in overcoming the developmental block, such as the level of osmolarity, the levels of sodium chloride (NaCL), glucose or phosphate levels, as well as significantly effect of altering the pyruvate: lactate ratio (Biggers, 1998). All of all, it seems that the *in-vitro* culturing conditions, that could be detrimental to the developmental capability of the embryos as well as failure of embryonic genome activation, are mechanisms that contribute to developmental block (Betts and Madan, 2008).

1.19 The Mitochondria and reactive Oxygen Species.

The mitochondria within the arrested egg have the same distinctive features as other mitochondria present in any other cell. They are known to have their own DNA that allows them to replicate independently of the cell cycle controlled via the nucleus. The mitochondria has two membranes surrounding its cytoplasm, the outer

and inner membrane with the inner membranes considered to be the main barrier as it is more impermeant. Two main cellular processes occur within the mitochondria, the tricarboxylic acid (TCA) cycle and the respiratory chain reaction. The mitochondria have a membrane potential of -150-180 mV which is independent of the cytoplasmic membrane and is negative to that of the cytosol (Duchen, 2000). This membrane potential is established due to proton transfer across its inner membrane creating an electrochemical proton gradient. The mitochondrial membrane potential highly influences cellular function as it produces ATP. It effects cytosolic Ca^{2+} homeostasis as it allows its movement along its electrochemical gradient into mitochondria (Smaili et al., 2000, Duchen, 2000).

1.19.1 Mitochondria in eggs

Morphologically mitochondria in eggs differ from those in somatic cells, they contain less cristae and are more spherical than the elongated ones present in somatic cells (Dumollard et al., 2004). Once primordial germ cells are formed they contain around 10 mitochondrion, they carry on and replicate throughout maturation, and by the end of the maturation process the replication ends. At this point they would have reached a number of several hundred thousand (Poulton and Marchington, 2002). The fertilised egg is supported by its mitochondria up to its blastocyst stage, however, fertilisation and development up to blastocyst stage is still possible even with suppression of mitochondrial DNA replication and translation (Huo and Scarpulla, 2001). The GV is the large nucleus present in the centre of the egg before maturation and the mitochondria during this stage will be present within the GV as clusters (Jansen, 2000). As soon as the meiotic spindle begins to form and starts to migrate the mitochondria leaves the GV and moves towards the animal pole (Nishi et al., 2003). During early stages of maturation the mitochondria will be found in close proximity to the endoplasmic reticulum which suggests an interaction between both organelles (Marchi et al., 2014, Sardet et al., 2007). In the metaphase II arrested egg the mitochondria will be accumulated around the nucleus at the centre and surrounding the meiotic spindle (Calarco, 1995).

1.19.2 Mitochondria and Fertilisation

The mitochondrial enzymatic processes are stimulated once Ca^{2+} oscillations start during fertilisation (Schomer and Epel, 1998, Campbell and Swann, 2006, Dumollard et al., 2008). Experiments have revealed that post sperm egg fusion and Ca^{2+} release, as a consequence oxygen consumption is initiated within the egg and

reaches a maximum (Dumollard et al., 2003). Even though sperm egg fusion allows the passage of sperm mitochondria into the egg cytoplasm, these do not participate in supporting embryo development as they get destroyed by the egg (Sutovsky et al., 2000). However, previous evidence that there are still medical cases that present due to inherited parental mitochondrial defects (Schwartz and Vissing, 2002). It seems that the fertilisation process drives a structural change in the mitochondria as its cristae become more prominent, and during fertilisation Ca^{2+} homeostasis and ATP production are highly influenced by the mitochondria (Dumollard et al., 2003, Dumollard et al., 2004). Poor embryo development and apoptosis is seen in mammalian eggs such as human, mouse and pig that have mitochondria that exhibit structural or functional defects (Van Blerkom et al., 1995). Another vital process that mitochondria control during fertilisation is the production of reactive oxygen species (ROS) (Morado et al 2013). Recordings demonstrated that a prominent ROS, hydrogen peroxide can potentially cause a higher fragmentation rate which is detrimental to the embryo's capability to develop (Liu et al., 2000, Kimura et al., 2010).

1.19.3 Mitochondria and Ca^{2+} signalling

The evolution of the eukaryotic cells over time has allowed development of toolkits specifically for Ca^{2+} detection and sensing. Those include specific proteins that have the ability to sense the changes in Ca^{2+} levels so signalling pathways could operate by detecting this change. Ca^{2+} channels operate as part of a homeostatic system to keep control of cytosolic Ca^{2+} level. The level of Ca^{2+} during inactive periods within the cytosol is known as the basal cytosolic Ca^{2+} and what allows this equilibrium in the long term is the interaction of the influx-efflux rates (Berridge et al., 2000, Rizzuto and Pozzan, 2006). Any organelle pumps or other buffering systems only affect the cytosolic levels transiently. Cytosolic changes of Ca^{2+} are caused by either entry from the extracellular space or release from intracellular storage sites, or a combination of both. At the end of a signal Ca^{2+} change the basal levels have to be rebalanced and this rebalance occurs at the expense of energy consumption through the action of pumps and antiporter (Rizzuto and Pozzan, 2006).

The endoplasmic reticulum has been long known to be the main intracellular Ca^{2+} store, however, almost all other intracellular organelles play a role in Ca^{2+} signalling such as mitochondria (Saris and Carafoli, 2005) the Golgi apparatus (Pinton et al., 1998), secretory vesicles (Mitchell et al., 2001)and many others. Much attention has

been paid to the mitochondria for its unique ability to handle Ca^{2+} in an energy dependant manner. Ca^{2+} strongly influences the function of many mitochondrial enzymes within the matrix such as dehydrogenases (Denton, 2009). For an example, mitochondrial enzymes present on the outer surface of the inner mitochondrial membrane (IMM) such as glycerophosphate dehydrogenase or the malate-aspartate shuttle and others. The simultaneous change of Ca^{2+} within the mitochondria associated with that of the cytosol has a wider scale of implications on the cell. The change within the mitochondria is in a larger range and this excess uptake from the cytosol can induce a bioenergetic failure through a number of mechanisms. Apoptosis and necrosis are means of organelle and cell death in cases of mitochondrial overload with Ca^{2+} and that is through opening of the permeability transition pore (PTP) and release of cytochrome c (Contreras et al., 2010, Calì et al., 2012, Pinto et al., 2015).

1.20 ROS and Effects on Embryo development.

Oxidative phosphorylation is a process on which the oocyte relies on in producing its ATP. This form of energy production depends on oxygen as a key substrate. Therefore, the total usage of oxygen has been used as an indicator of the eggs metabolic activity (Leese, 2003). During the early cleavage cycles the mitochondrial activity is thought to account for at least 30% of the total oxygen consumption, this figures doubles as the embryo gets to the blastocyst stage (Trimarchi et al., 2000). Oxidative energy production is not free of risk as it comes at a cost of reactive oxygen species production, more specifically the superoxide anion and the hydroxyl radical. Once the superoxide anion is catalysed via dismutase it produces hydrogen peroxide and in presence of metal ions such as iron it can yield metal-centred oxygen radicals, these species are detrimental to proteins and DNA (Guérin et al., 2001). Early studies have revealed that overproduction of ROS in fertilised eggs can cause developmental failing (Johnson and Nasr-Esfahani, 1994). Aitekn et al in 1989 has connected the generation of these oxidative species to the arrest of cell division and loss of cell function (Aitken et al., 1989). On the other hand, ROS production is essential for normal cellular processes to function and contribute positively in growth and development (Hardy et al., 2001). Necessary gene activation has been observed in response to alteration in the level of ROS production, as a result, it is suggested that it's the correct balance between the production and clearing of ROS that is essential not the mere presence of ROS

(Burdon, 1996). During sperm penetration in bovine eggs an increase in ROS production has been recorded, and this increase is known as the respiratory burst during fertilisation (Nasr-Esfahani and Johnson, 1991). This increase in ROS production via increased mitochondrial activity has been suggested to be caused by the Ca^{2+} changes that occur during fertilisation. Depending on the level of oxidative stress it can lead to either necrosis or apoptosis (Burdon, 1996). Programmed cell death (PCD) could be caused by increased levels of oxidative stress as a result of raised levels of H_2O_2. Reports have shown elevated levels of H_2O_2 in fragmented human embryos (Betts and Madan, 2008). In a recent study early embryonic developmental arrest has been directly related to mitochondrial dysfunction (Thouas et al., 2004), moreover, sub-lethal doses of photosensitisation to mitochondria have shown delayed negative effects. The detrimental manifestations of sub-lethal mitochondrial damage appeared at later stage where the embryos were aborted as a result of implantation failure (Thouas et al., 2004). However, in the same way that the ROS can be present at any site in the embryo, there are defensive mechanisms that are present at various locations in the cell. In embryos ROS can be produced in mitochondria as well as the cytosol, protective enzymes found at these sites are the mitochondrial superoxide dismutases (Mn-SOD) and its cytosolic version (Cu, Zn SOD). The free radicals are converted into H_2O_2 which in turn is removed via GPX in cytosol and mitochondria (Guérin et al., 2001).

1.21 Hyper, A Novel Intracellular Hydrogen Peroxide detector.

Since the 2-cell block phenomenon has been identified (Goddard and Pratt, 1983), unravelling the factors that could lead to it has led to a conclusion that reactive oxygen species particularly hydrogen peroxide are involved as one of the main causes for this phenomenon of cell damage *in-vitro* (Aitken et al., 1989). Many studies have been designed in an attempt to measure the level of H_2O_2 in individual living mouse eggs and embryos (Nasr-Esfahani and Johnson, 1992, Lopes et al., 2010). The basis of most of these studies was the use of 2, 7-dichlorodihidrofluorescein diacetate (DCHFDA) AM dyes as they are readily permeant into the cells where they get trapped due to the action of the intracellular esterases that hydrolyse these compounds yielding DCHF (Chen et al., 2010b). The magnitude of fluorescence resulting from oxidation of the dye by H_2O_2 seems to be linearly related to the level of H_2O_2 present in the cell, therefore it was suggested that the level of fluorescence could be used as a direct estimate of intracellular

H_2O_2. This has been shown in mouse strains that were more prone to the blocking such as the MF1 as well as the non-blocking strains such as F1 (Nasr-Esfahani et al., 1990a).

The OxyR gene has a vital role within *E.coli* for its ability to activate genes that encode scavenging enzymes for reactive oxygen species, the conformational changes that occur to the OxyR protein when transformed from its reduced state to the oxidised state provides the means of it sensing an oxidative stress signal and therefore stimulates the ability of the cell to switch on the defence mechanisms (Christman et al., 1989). Therefore the design of this novel probe was based on the known properties of the *E.coli* protein known as OxyR and this protein is a specific H_2O_2 sensor while remaining unaffected by other reactive oxygen species (Storz and Tartaglia, 1992). The novel probe known as HyPer is claimed to be a specific probe for detecting hydrogen peroxide changes within living cells, the main component of this probe is the H_2O_2-sensing protein known as OxyR that is found in prokaryotic cells, and the circularly permuted yellow fluorescent protein (cpYFP) that has been incorporated into this regulatory domain (Belousov et al., 2006). Within the cells the oxidised HyPer has shown the ability to reduce back to its initial state which is a main feature of the wild type OxyR. The fluorescence increase marking the reaction of HyPer with hydrogen peroxide comes down to its initial baseline several minutes following the H_2O_2 burst (Belousov et al., 2006).

1.22 Project Aims

In this dissertation the intention was mainly to investigate the ability of PLCζ protein to rescue cases of failed fertilisation. I developed a protocol that would be applicable

in IVF clinics to treat cases of failed fertilisation in certain cases of infertility. The first aim has two facets to its purpose. The first was to create a mouse model of male infertility in our laboratory. The model results in a number of phenotypes of sperm in terms of their ability to induce Ca^{2+} oscillations in mouse oocytes. The next step further was to rescue the oocytes with PLCζ and asses the level of pre-implantation development. In light of the fact that currently several IVF clinics are using a number of chemical agents in rescuing cases of failed fertilisation, a comparison of PLCζ to other egg activators was set as an aim as well. The second key aspect was to construct a complete protocol that would develop a precise method of injecting PLCζ in terms of identifying media properties, injection needle characteristics, and how to inject a therapeutic dose of this protein. This protocol created is designed to be applicable to human eggs in IVF clinics.

The final chapter was designed to investigate causes of poor development after activation, by examining if there is an excess of reactive oxygen species generated at fertilisation. The hypothesis was built around the fact that Ca^{2+} changes during fertilisation influences mitochondrial activity and that in turn could be in a positive (ATP production) or a negative (excess ROS) mode, depending on the frequency of oscillation. Reactive oxygen species were studied using a novel hydrogen peroxide sensor for the first time in eggs. A final separate chapter will summarise the data in all results chapters providing a detailed discussion of all findings in this thesis.

Chapter 2

MATERIALS AND METHODS

2 Material and Methods.

2.1 Health and safety and legal procedures

All of the chemicals were handled and stored according to the manufacturer's regulations recommended and all of the PLCζ RNA was handled as required by the Genetic Manipulation Advisory Group (GMAG). All of the experimental work was done in accordance to the regulations of the control of substances Hazardous to Health (COSHH) and Cardiff University laboratory regulations. Human egg work was started after obtaining a licence from the Human Fertilisation and Embryology Authority (HFEA). Human eggs were used to investigate the role of sperm PLCζ in human oocyte activation, a research licence was issued (R0161/2/c). All donated human eggs for scientific research were handled and treated according to HFEA regulations and guidelines.

2.2 Laboratory reagents and animals

All chemicals and reagents were obtained from Sigma UK unless stated otherwise and were of the best grade available such as 'embryo tested' or 'cell culture tested'. The cRNA constructs were kindly supplied by Prof. Tony Lai and made by Michail Nomikos. In summary, the cRNA that was used in some experiments was synthesized using a well-defined protocol, the open reading frame of mouse PLCζ was used for this purpose. Measuring the absorbance at 260nm of each batch of cRNA made in a 500µl quartz cuvette and with the aid of a specific equation quantification of the product was possible. *In-vitro* protein expression was done to check the cRNA and that was done in a rabbit reticulyte lysate system (Promega). Previous publications from the group have details of this process (Saunders et al., 2002, Nomikos et al., 2005, Swann et al., 2009).

2.2.1 Production and purification of recombinant protein

Protein was also made by Michael Nomikos. In short the recombinant protein produced had a NusA tag to increase solubility and 6x-Histidine (6x-His) tag to allow purification. The relevant pETMM60 plasmid was transformed into Escherichia coli (BL21-CodonPlus (DE3)-RILP, Stratagene, CA, USA). The transformed *E. coli* were then cultured at 37°C before the temperature was reduced to 16°C for 18 hours where protein expression was induced. Once cells had been harvested they were re-suspended they were sonicated. The recombinant protein was then purified using nickel-nitrilotriacetic affinity chromatography matrix (Ni-NTA Agrose; Qiagen) which

selects for His tagged proteins. The protein was eluted using 250mM imidazole then dialysed overnight which after it was concentrated. Recombinant protein was either used immediately, kept at 4°C for short term storage or snap froze and stored at -80°C for long term storage.

2.2.2 Mice

Female MF1 mice were bought from Harlan at about 6 weeks of age. Male mice are C57/CBA F1 hybrid that were obtained from (Harlan, UK Ltd.). Male mice were used for mating as well as retrieving sperm used for IVF and ICSI (Intra-cytoplasmic Sperm Injection).

2.2.3 Hormone preparation for super-ovulation protocol

Hormonal treatment was used to superovulate female MF1 mice which were around 6 weeks old at the time. Hormones that were used for this purpose were given at two stages and are pregnant mare serum gonadotrophin (PMSG) (PMSG; intervet) and human chorionic gonadotrophin (hCG) (HCG; intervet). The first stage of ovulation induction utilises PMSG at 2-4pm (day 1) as it mimics the action of the follicular stimulating hormone (FSH) and it stimulates the growth and development of the ovarian follicles and is given as a concentration of 5-7 IU. This first injection is followed 48hrs later by a second intra-peritoneal injection of hCG at 6pm of day 3 as it mirrors the action of the luteinising hormone (LH) by promoting maturation of the follicles and allows their release, the concentration used for this particular hormone is 10 IU. 15hours post hCG injection the mice were culled for tissue harvest, oviducts in particular.

2.3 Fire pulled glass pipettes

Before performing any experiment there are tools that need to be made such as the glass pipettes that are used to transfer the eggs from one solution to another or from one dish to the other. The transferring process is carried out by attaching a changeable mouth piece which is a sterile 1ml plastic syringe with the plunger taken out to plastic tubing, the wide end of the glass pipette is connected to the tube via a plastic tube connecter that has a cotton wool plug. This fine bore glass pipette is prepared in the laboratory by holding the narrow end of a disposable glass pipette over a small flame of a Bunsen burner. The pipette is rotated whilst it is over the flame to ensure even heating of the glass, when the glass starts to melt a sudden and fast pull from one end of the pipette is done. Breaking the pipette at

approximately 5cm from its origin is the next step and this will produce an opening with a diameter slightly larger than that of the egg (>80µm), to allow smooth pick up and release of eggs. The glass pipette was changed frequently during any experiment.

2.4 Collection of eggs

The female MF1 mice that have been super-ovulated by intra-peritoneal injection of two hormone doses were sacrificed by the use of schedule 1 for humane animal killing. The mice were sacrificed in the Joint Biological Services (JBIOS) facility at Heath Park, Cardiff University in accordance with HM Home Office regulations. The CO_2 chamber was used for the first stage until the mice were believed to have ceased spontaneous breathing then cervical dislocation was followed up to ensure death. Subsequently a simple dissection was done to retrieve the oviducts as shown in Fig.2.1. The oviducts were placed into warm M2 media (Sigma-Aldrich) and carried over to the lab to release the eggs. Under a dissecting microscope the ampullas were pierced using a 25 gauge needle while the oviduct being held by a forceps, the egg-cumulus complex gets releases from the tear site into dish that contains pre-warmed hyaluronidase with a concentration of 300µg/ml in M2 media. The egg-cumulus mass remains in the media with hyaluronidase enzyme for no longer than 5 minutes which allows time for denuding the eggs from the surrounding cumulus cells (Lawrence et al., 1997). Fig.2.2 shows the eggs before and after treatment with hyaluronidase, following this the eggs are washed in at least 3 drops of M2 media to remove any traces of carried over media with hyaluronidase. After this step the eggs are kept in 100µl droplet of M2 covered by embryo tested mineral oil and left to recover at least for ten minutes at 37 degrees before any experimental procedure being carried on them. In some experiments I have used GV eggs (eggs with germinal vesicles). Briefly, unprimed mice were used for this purpose (i.e no hormones were injected), oviducts were collected as mentioned before and with the use of a forceps and a 25 gauge needle the ovary was cut into fine pieces. With the use of a fine pipette, oocytes with a GV were collected and the cumulus cells surrounding them were mechanically removed by the use of the fine pipette as well. The oocytes were maintained in the GV state by incubating them in M2 media containing 250µM IBMX and covered with embryo tested mineral oil and kept at 37 degrees until injection or further treatment.

2.5 Human Eggs

Couples from IVF Wales clinic or Neath Port Talbot who were engaged in this project had a briefing about the aims of the research conducted and were given a consent form that described the project in detail. Only those who gave written consent for use of their unfertilised eggs were involved in this project. From both clinics eggs that had failed to fertilise after IVF or ICSI and in some instances GV eggs were used as well. From IVF Wales clinic, eggs were collected personally and eggs from Neath Port Talbot were delivered in a heated flask to maintain the temperature throughout the journey. A handover protocol was followed during receiving and delivering the eggs.

2.6 Sperm collection and preparation

After male mice were sacrificed using the CO_2 method followed by a confirmation with cervical dislocation, dissection to the lower abdominal region was done. When the testis per side was exposed the epididymis was collected by cutting the vas deferens near to the caudal epididymis using a pair of clean fine scissors and a forceps and kept in pre-warmed M2 media, this procedure was carried out in the Joint Biological Services (JBIOS) facility at Heath park, Cardiff University in accordance with HM Home Office regulations. The collected epididymis were carried over to the laboratory where sperm were squeezed from the caudal region of the epididymis into a dish containing about 1ml of and pre-warmed T6 media (Quinn et al., 1982). The T6 media after being prepared in stock and kept at 4 degrees, when to be used addition of 15mg/ml of bovine serum albumin (BSA) and sterile filtered after which it is kept overnight in a 5% CO_2 incubator at 37 degrees to equilibrate to a pH of 7.6. The sperm, when released into the pre-equilibrated T6, are given a period of around 5 minutes to swim out then made up to a total of 5ml to achieve a required concentration to be used for in vitro fertilisation. The tube containing T6 with sperm is returned into the incubator to allow for capacitation. If the sperm is to be used for ICSI experiments they are aliquot into 250µl aliquots and stored in -80 freezer until further use.

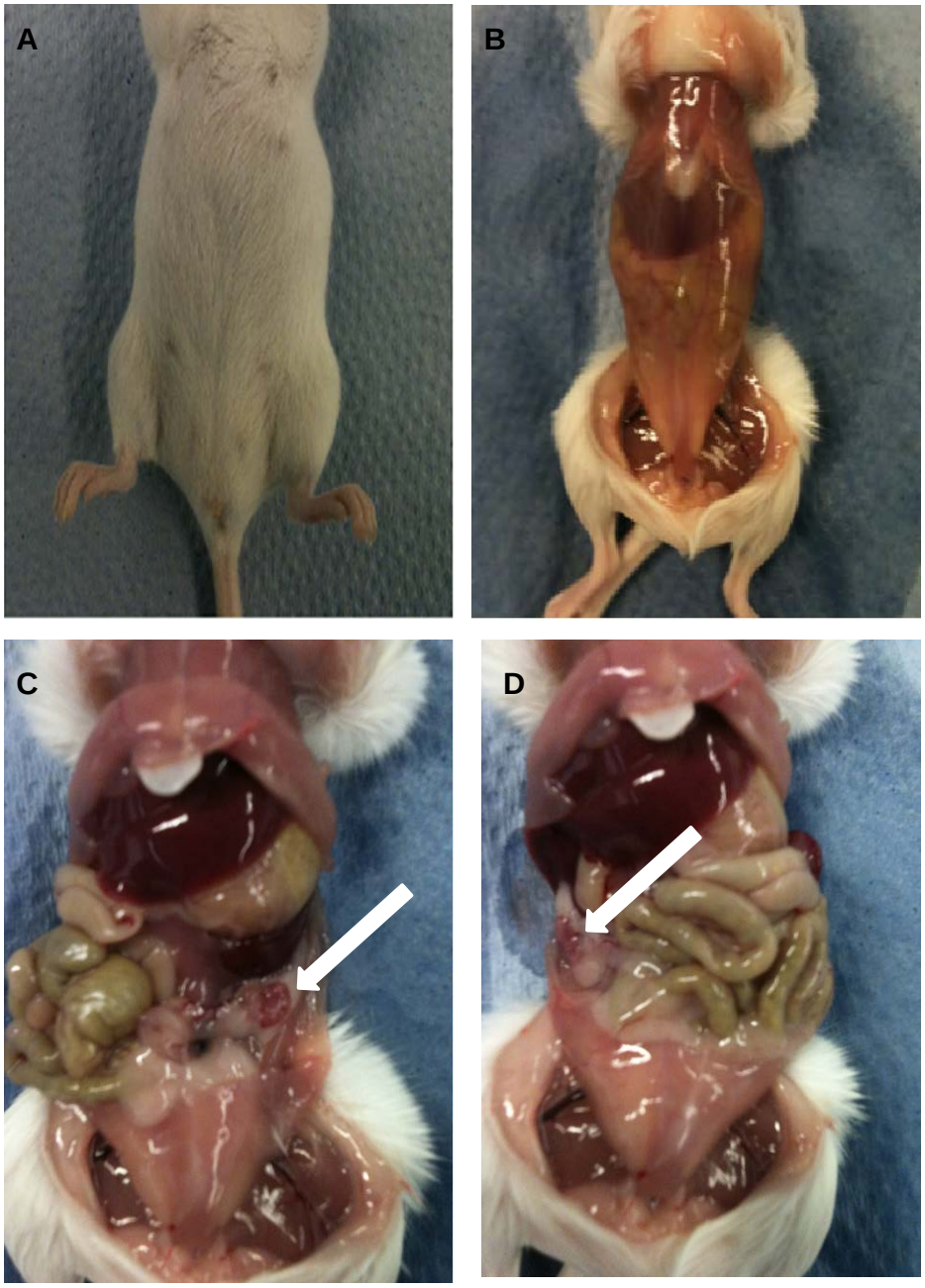

Figure 2.1 Dissection of MF1 female mouse post superovulation.

A Mouse culled via schedule 1 and positioning for dissection. B, Skin dissection and revealing abdominal peritoneum. C, D Peritoneum retraction and gut deflection to either side to reveal ovary and oviduct that will be cut out.

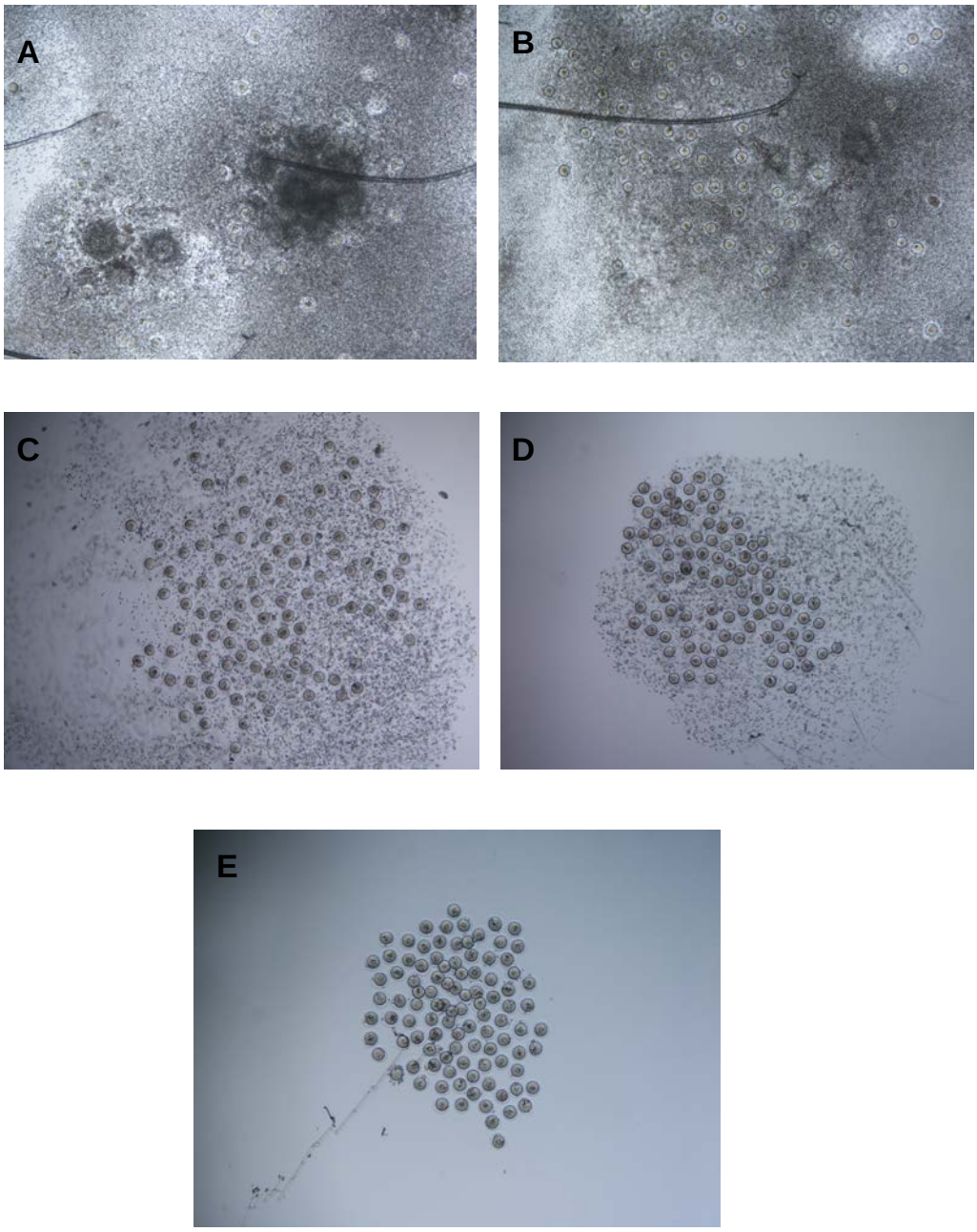

Figure 2.2 Hyluronidase treatment of cumulus masses.

A, Cumulus masses after release from oviducts into the hyluronidase drop. B Eggs starting to dissociate from the mass. C, Eggs dissociating for the surrounding cumulus cells. D, E Free eggs are washed through several drops of M2 to remove

traces of hyluronidase and cumulus cells which after eggs are kept in an M2 drop covered by mineral oil until further use.

2.7 Chemicals that alter intracellular Ca^{2+} levels and/or parthenogenetically activate MII eggs

Ca^{2+} transients can be stimulated and detected in eggs after physiological sperm attachment and after ICSI. However other non-physiological methods to increase intracellular Ca^{2+} have been proposed either in a repetitive or non-repetitive manner. When eggs are activated without mating, or by other methods not involving fertilisation, this process is known as parthenogenesis. I have used the Ca^{2+} ionophore which was prepared as a 10mM stock in DMSO and a final concentration of 10µM was used in parthenogenetic activation protocols. During the first hour of fertilisation or activation the emission of the second polar body takes place to release half of the chromosomes making the egg ready to fuse with the male pronucleus that contains the other half of the genetic information required for optimal development. However, it is much more accurate to say that no actual merging takes place but when the membranes of both pronuclei dissolve, the chromosomes of both contributors align on the spindle apparatus at the equator. In cases of parthenogentic activation to ensure proper cell cycle progression this event is prevented by the presence of cytochalasin B or D (CB or CD). CB is a cell permeable mycotoxin, it strongly inhibits network formation by actin filaments and therefore blocks the contractility of microfilaments and cytoplasmic division. A 5mg/ml stock was prepared in DMSO and when required it was diluted to a working concentration of 5µg/ml

2.8 Media preparation

M2 media obtained (from Sigma Aldrich) was aliquot into 10 ml test tubes and kept in the fridge. Every working morning one tube is taken out and placed in the desktop heated block for the use of that specific day and any remaining media will be discarded at the end of the day. Potassium chloride (KCL) Hepes buffer (100mM KCL and 20mM Hepes) were prepared to be used as a diluting media for reagents to be injected into eggs. Chelex 100 beads were used to eliminate any ions that were present; pH was calibrated and ensured to be at 7.5. Concentrated Hepes buffered potassium simplex optimized media (HKSOM) was made as a 10x stock and kept in the -80 freezer. 500 ml of sterile water was added to the components shown in Table 2.1 to make the required stock (Erbach et al., 1994). HKSOM is

prepared from the concentrated stock (with the use of 1ml) by adding 7.5 ml of sterile water and Ca^{2+}chloride (10µl of 1.7M $CaCl_2$) and 10 µl of 0.2 M glucose, the pH is adjusted to 7.4 by adding 20-30 µl of sodium hydroxide. The osmolarity is confirmed for each preparation made before commencing experiments to 280mOsm/kg by using a freezing point osmometer (Gonotec Osmometres-Osmomat 030, Germany). However, different versions of this media were made according to the experiment intended.

2.8.1 Low sodium HKSOM

The idea behind the use of a media that has almost all of the sodium being omitted from it is to create a hyper-osmotic media (350-400mOsm/kg) and it was used in some of the ICSI experiments. It was prepared in the same manner as the full HKSOM with replacing the sodium salts with their choline correspondents in order to maintain a higher osmolarity.

2.8.2 Sr^{2+} HKSOM preparation

In order to use strontium chloride as a parthenogenetic activator for eggs, Ca^{2+} free HKSOM was prepared in the exact same way as the regular HKSOM with omitting the Ca^{2+}chloride component. The Ca^{2+} component was replaced by Sr^{2+} with a concentration of 5-10mM, as it has been reported that this concentration with exposure duration of 2.5 hours in mouse eggs has the best pronuclear development rate, blastocyst formation and cell number in parthenotes (Ma et al., 2005). This media has been used to parthenogenetically activate MII eggs, 2µM cytochalasin B was used in the media to prevent extrusion of the second polar body and produce a diploid embryo instead of a haploid embryo (Meinecke-Tillmann and Meinecke, 1980). Therefore the eggs are first washed three times in Ca^{2+} free HKSOM to ensure all traces of Ca^{2+} are eliminated then the eggs are incubated in this media for about 2-2.5 hours and then removed and allowed to recover in regular HKSOM before culturing.

Chemical	10x stock (stored at -80 after filter sterilisation)		Working media (1x) pH 7.4		MW
	g/500ml	Ml/500ml	mM	g/l	
NaCl	27.75		9.5	5.55	58.44
KCl	0.925		2.5	0.185	74.56
KH2PO4	0.238		0.35	0.0476	228.2
NaPyruvate	0.11		0.2	0.022	110.04
Streptomycin sulphate	0.25			0.05	
Penicillin G	0.315			0.063	
EDTA	0.019		0.01	0.0038	416.2
MgSO4. 7H2O	0.246		0.2	0.0493	246.48
Na Lactate (liquid)		7.37	10.0		112.1
NaHCO3	1.68		4.0	0.336	84.01
HEPES	23.8		20.0	4.76	238.31
Phenol red	0.055				354.38
L-glutamine	0.73		1.0	0.146	146.15

Table 2.1 Different components of HKSOM medium.

HKSOM medium in both forms, the 10x stock which is stored at -80 until further use after filter sterilisation and the working 1x prepared media.

2.9 Microinjection of mouse eggs

The microinjection needles are pulled on a daily basis first to prevent any contamination to the tips and to ensure a sharp tip for every needle used. Borosilicate glass capillaries are used (Harvard Apparatus Ltd. 1.5 mm outer diameter and an inner diameter of 0.86 mm). These glass capillaries are characterised by having an internal filament which increases the capillary action and eases the back filling process. The glass capillaries are pulled on a vertical pipette puller (Model P-30; Sutter Instruments), the needles are backfilled with < 1µl of the injection solution using microsterile micropipettes (Ependorff). The needle is then fitted to a holder (ME2H25FW, World Precision Instruments) that has a silver wire which goes down through the needle. The needle holder has a side port that is connected to a picopump pressure injector (model PV820, World Precision Instruments) via a silicon tube. The holder then is securely attached to another clamp that is connected to a preamplifier of an electrometer (IE-25A, Warner Instrument Corporation). The preamplifier, needle holder and the holding pipette are mounted on hydraulic manipulators (Narashige) which are in turn fixed to an inverted microscope (TE2000, Nikon).

The injection needle is directed by a manual control towards the base of the dish that contains the eggs to be injected. Eggs are held in position with the use of suction by a holding pipette that has been previously pulled from G-100 TF glass (Harvard Apparatus Ltd) using PN-30 pipette puller (Narashige). The holding pipette is attached to a syringe system (Cell Tram Air 5176, Eppendorf), each egg is held individually by the holding pipette through negative pressure which allows the egg to be sucked onto the holding pipette. The injection needle is driven towards the egg piercing the zona pellucida and once it reaches the cytoplasmic membrane a small electrical oscillation using the electrical amplifier is delivered. This oscillatory action allows the injection pipette to penetrate the plasma membrane without damage. Microinjection of the solution required is delivered by applying high pressure pulses to the back of injection needles (usually 20 psi for approximately 0.5seconds). The injected solution is estimated by the cytoplasmic displacement caused by the injection itself, which is estimated to be 3-5% of the egg cytoplasm volume (Larman et al., 2004). Wherever PLCζ recombinant protein or the cRNA were to be microinjected they were diluted in Hepes buffered injection buffer (KCL Hepes) and where Ca^{2+} was to be measured the diluted protein or cRNA was mixed with equal

parts of a Ca^{2+} indicator. The solution was mixed and spun to the bottom of a 0.5ml tube using a benchtop centrifuge (Spectrofuge 24D, Jencons) prior to microinjection. Injected agents will be elaborated upon in the corresponding results sections. In cases where multiple agents are required to be injected such as (Rhod dextran dye and mouse PLCζ RNA) a co-injection protocol would be carried out to reduce the potential damage that may be caused to the egg by multiple injections (Fig. 2.4). Different experiments have differing protocols so each protocol will be discussed in its related result section.

2.10 *In vitro* Fertilisation (IVF)

IVF is carried out around with eggs 16-18 hours after hCG administration. Sperm that has capacitated in the incubator and prepared as mentioned previously is used for this experiment. When Ca^{2+} changes during IVF are intended to be recorded the eggs are preloaded with 2μM of PE3 AM dye (Fura2 leakage resistant dye) as they are incubated for 30 minutes in the dye, washed in at least 3 drops of M2 to remove any dye trace, then allowed to recover in an 100μl M2 drop covered by mineral oil. Removal of the zona pellucida was done by a brief exposure to acidified Tyrode's solution, in order to stabilise the eggs during the addition of the sperm. Sperm egg fusion can lead to movement and that will affect the Ca^{2+} traces recorded. The zona free eggs are washed in three drops of M2 media before being placed in a dish with 1ml of HKSOM or M2 and this process allows the eggs to stick to the glass cover slip of the dish and reduce any movement. The recording is initiated and is allowed to proceed for about 15 minutes first to ensure that the eggs are firmly attached to the bottom of the dish and secondly the base line recording of Ca^{2+} has been stabilised. A 20μl of sperm preparation is added to the dish with eggs and direct visualisation of the eggs is done to ensure enough sperm is available around the eggs to ensure fertilisation completion and then the recording is continued. When an IVF run is carried out to obtain embryos and follow up their development, dye loading and acid Tyrode's steps are omitted. Eggs are placed in a dish with 1 ml of HKSOM and covered by mineral oil and 20μl of sperm preparation is added. After a period of 1 hour the eggs are washed in three drops of M2 and cultured.

2.11 Intracytoplasmic sperm injection (ICSI)

The first generated mouse offspring from using ICSI technique was shown by Yanagimachi in 1995 (Kimura and Yanagimachi, 1995). However, the micromanipulation technique itself was developed by Atsushi Mimatsu and colleagues and it utilised the piezoelectric effect (Kimura and Yanagimachi, 1995). The use of piezo in mouse ICSI allowed the survival rates to reach 100% when it rarely exceeded 50% when conventional methods were used. Naoko Yoshida & Anthony CF Perry provided a detailed description of the procedure (Yoshida and Perry, 2007), therefore I will summarise the technique that is used for the ICSI experiments included in some parts of this work.

2.11.1 ICSI Microinjection needle preparation

Borosilicate glass capillaries (G-100 TF glass) (Harvard Apparatus Ltd) are pulled on a micropipette puller using PN-30 (Narashige). On a microforge the tip of the pulled pipette is broken by applying a brief heat (current) pulse producing a flat sharp end with a diameter of 6.5-8.5μm. At a distance of about 3-5 mm from the tip a bend of about 30^{o} is made by gradually heating the glass pipette and stored in a Petri dish for later use. Figure 2.3 shows various instruments used in the make f the pipettes.

2.11.2 Preparation of the workstation for micromanipulation

The work station for micromanipulation is shown in Fig. 2.4. A pipette egg holder is placed on one side while the microinjection needle is mounted on the other side after being backfilled with Flourinert (FC-770) and ensuring no air bubbles between the oil and flouirnert interface. The dish preparation will depend on the type of experiment carried out and will be described in relevant chapters. However basically it will contain a drop of M2 media or/and other type of media, expelling several drops of fluorinert in order to wash and lubricate the interior for a smoother control of the material to be injected. Eggs are placed in the M2 drop ready to be injected.

2.11.3 The injection

The material to be injected either sperm or PLCζ is either placed in a separate drop or within the drop that contains the eggs. A sperm or a column of PLCζ is withdrawn into the injection pipette and then advanced towards the egg. Having the MII plate at 6 or 12 o'clock position and the egg plasma membrane in sharp focus, the microinjection needle is advanced towards the zona pellucida and an application of

repetitive moderate piezo pulses will allow the needle to advance into the perivitelline space. The microinjection needle is then pushed towards the plasma membrane until it produces a deep invagination into about 90% of the egg diameter. A gentler single piezo pulse is given and the plasma membrane will relax and retain its original shape then the material to be injected can be deposited by applying positive pressure. The needle is withdrawn from the egg cytoplasm smoothly and the plasma membrane should appear intact. After finishing the injections, the eggs are examined and the surviving eggs are transferred into a drop of M2 and placed on a bench top incubator to be examined later for pronuclei development and further culture. If the aim of the injection was to image Ca^{2+} changes after injection of sperm or PLCζ the eggs are taken directly to a heated stage on Nikon microscope based fluorescence system.

2.12 Mouse embryo culture and development

Mouse embryos were cultured *in-vitro* in drops of freshly reconstituted KSOM (Merck Millipore). The KSOM is equilibrated the night before culture in 5% CO_2 incubator at 37 degrees after being covered by mineral oil. The size of the drops are usually governed by the number of embryos in each drop, usually around 2-3µl of media per embryo. Where embryos were followed by time lapse imaging or there was a need to take an image on a regular basis such as in case of examining cell cycle division timings a Lumascope (Etaluma Inc, USA) has been used for this purpose. The Lumascope provides fluorescence and bright field microscopy in an inverted microscope and its compact size enables working in challenging locations including inside incubators. Easy routine inspection of eggs from within the incubator with its high-quality imaging minimises any photo damage that otherwise would have to be taken outside under a regular microscope. The Lumascope fits in incubators, therefore it provided a reasonable mean to image regularly (time lapse) while maintaining the optimal incubation environment. With just the power from the USB port of a laptop required, it provides a convenient portable fluorescence microscopy workstation. With this USB connection the images and the time lapse series are recorded directly on the computer. Analysis of data was done post experiment completion offline using Image J and Sigma plot software.

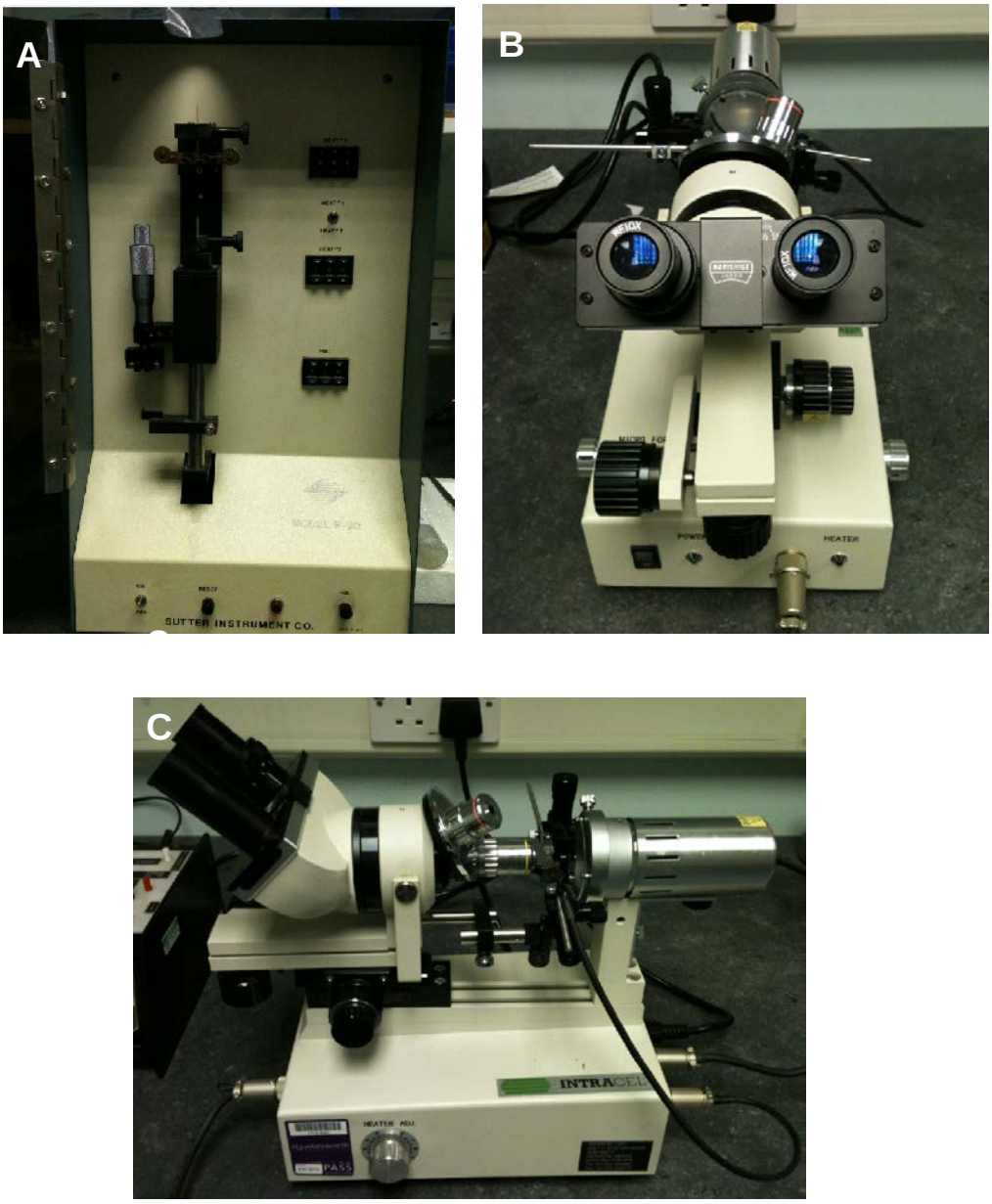

Figure 2.3 Instruments used in the making of various pipettes.

A, the injection pipette puller (SUTTER Instrument Corp P-30). B, C The holding and ICSI pipette beveller (Intracel, Microforge, MF-900-Narishige-Japan).

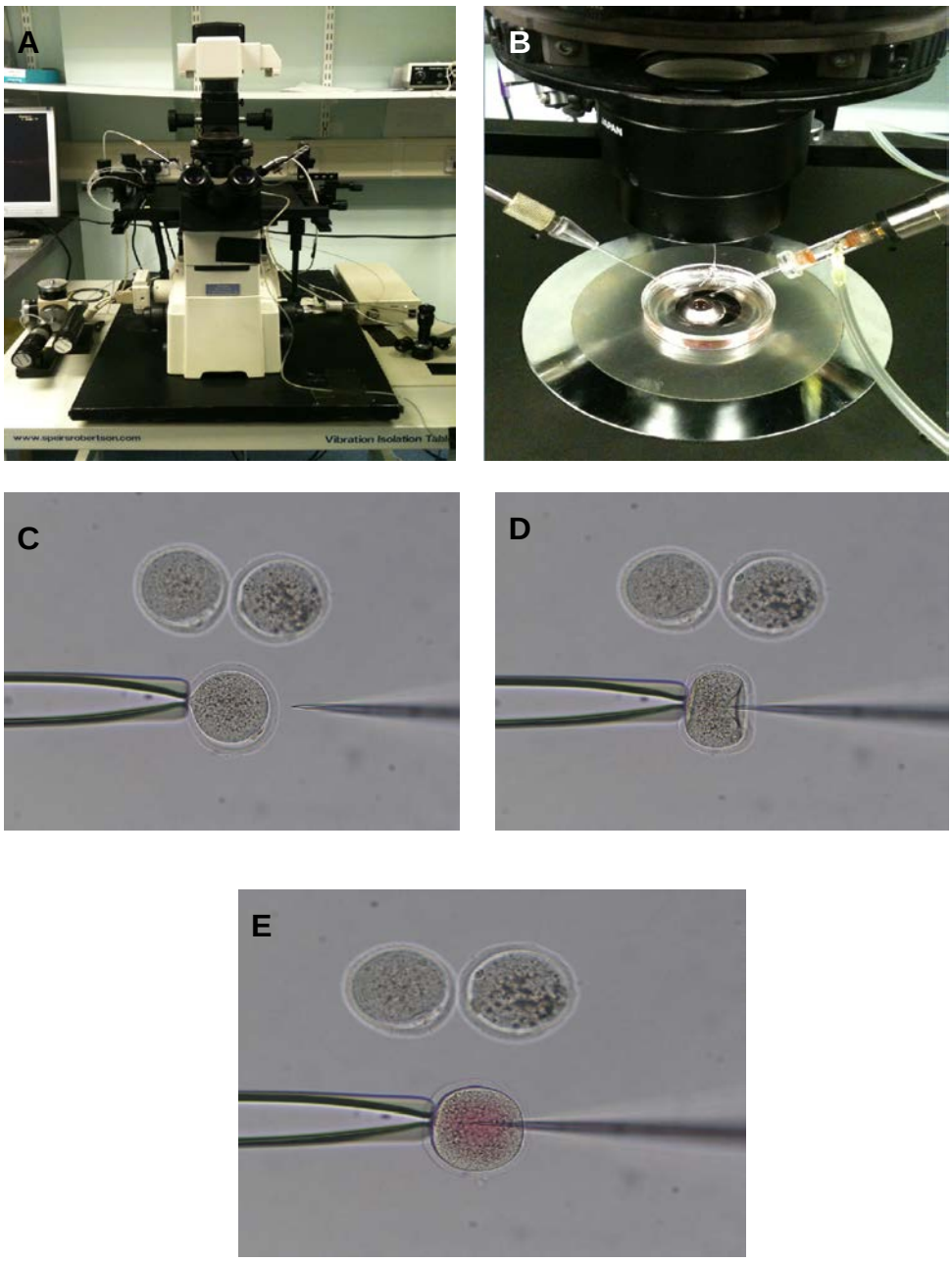

Figure 2.4 The micro-injection system.

A, The microinjection system, the inverted microscope (Nikon ECLIPSE TE2000_S, Japan) fitted with the pipette holders. **B**, The injection dish placed on the system, holding pipette to the right and the injection pipette to the left. **C, D, E**, Images of egg microinjection process, egg held in position by the holding pipette while the injection pipette advances through the zona pellucida and a bolus dose of Rhod-dextran dye microinjected (Rhod-dex used to show material delivery inside the egg).

2.13 Ca^{2+} Indicators

A range of fluorescent Ca^{2+} indicators have been used to measure Ca^{2+} fluctuations (Table 2, 2). The fluorescent Ca^{2+} probes were either dextran linked or AM dyes, dextran linked dyes were microinjected directly into the cytoplasm ahead of imaging alone or in combination with PLCζ protein or its corresponding cRNA.

2.13.1 Dextran linked Ca^{2+} Indicators

Rhod-2 Dextran (Rhod dex) and Oregan Green BAPTA Dextran (OGBD) are examples of this category of indicators that has been used in some sections. These dyes are dextran linked yielding an indicator that is retained within the cytoplasm for long periods of time without compartmentalisation. These hydrophilic polysaccharides are biologically inert, water soluble and have low cell toxicity. Rhod dextran has been preferred in a lot of experiments for measuring Ca^{2+}, its high Kd (750nm) reduces Ca^{2+} buffering effects and it has a large dynamic range. It also has the advantage of being excited by green light which is less phototoxic to eggs and upon excitation it emits a red light which is far from the autoflourescene emitted by the cell.

2.13.2 AM indicators

In contrast, the cell-permeant acetoxymethyl (AM) esters are loaded passively into the egg cytoplasm, where they are cleaved to cell-impermeant products by intracellular esterases. The non-ionic and nondenaturing detergent PluronicR F-127 are frequently added to help disperse the indicator in the loading medium. After an incubation period of 30 minutes at 37°C, the cells are washed across at least 4 drops of M2 which is a serum-free medium as serum may contain esterase activity. The loading medium (M2) is also free of amino acids or buffers containing primary or secondary amines because aliphatic amines may cleave the AM esters and prevent loading. Cal-520 is an example of this type of Ca^{2+} indicators that has been in sections of this work, it has been used on both mouse and human eggs, loading time for human eggs is 45 minutes. Others are Fura-2 leak resistant AM dye (PE3).

Ca^{2+} Indicator	Excitation (nm)	Emission (nm)	Kd (nm)
Cal-520 AM By AAT Bioquest	492	514	320
Fura-2 Leak Res AM (PE3) By TefLabs	335	495	145
Rhod dextran By Life Technolgies	552	581	750
Oregon Green BAPTA dextran (OGBD) By Life Technolgies	494	523	170

Table 2.2 Various Ca^{2+} indicators used throughout the project.

Different dyes used for measuring Ca^{2+} changes within the eggs with their excitation, emission wavelengths and Kd values.

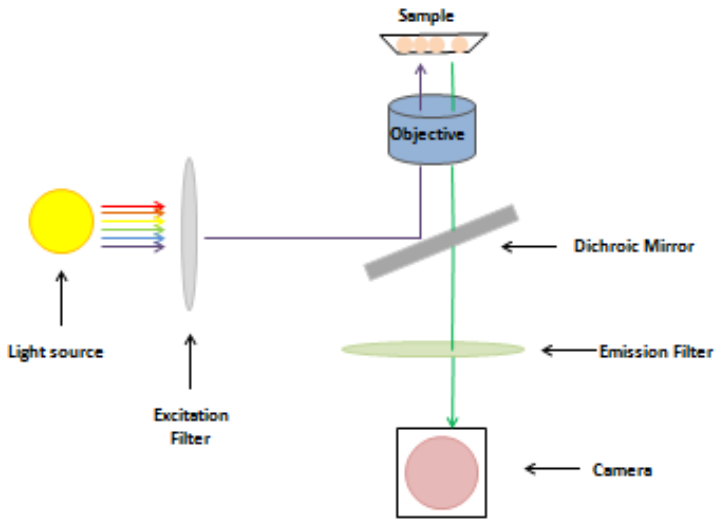

Figure 2.5 Schematic diagram of Epi-fluorescence using an inverted microscope system

2.14 Measurements of intracellular Ca^{2+} changes

Within the laboratory there are different imaging systems and the system to be used is defined by the experimental design and the purpose.

2.14.1 A Nikon microscope based fluorescence system

An inverted epifluorescence microscope (Nikon Eclipse Ti-U microscope) that has a heated stage (Warner Instruments) is used for most of the fluorescence imaging recordings (Fig 2.5). The heated stage ensures that the eggs are maintained at 35-37 degrees during this process. The light source for this system was either a white halogen lamp or an LED lamp (Optolite LED Lite, Cairns research Ltd), the light from the source passes through a filter of the appropriate wavelength. The filter allows only the light that is within the excitation spectrum of the fluorescent protein in the egg to pass through the objective to reach the sample after reflecting off a diochoric mirror. When light hits the sample the fluorescent dye or protein within the egg absorbs it and emits a light that has a bigger wavelength which is within the emission spectrum. The cooled CCD camera (CoolSNAP HQz, Photometrics) which is connected to the computer and In Vivo software collects the emitted light that has passed back through the objective and via the diochroic mirror and the emission filter. An optical filter changer (Lambda 10-3, Sutter Instrument Company) switches filters at set intervals and provides a means of automatically changing the filters, allowing more than one fluorescent channel to be detected during a given experiment. The objective used for imaging of Ca^{2+} fluorescence was a 20x (0.75 N.A, Plan Apo) objective, only in some experiments where human eggs were imaged a 10x (0.50 N.A, Flour) objective was used. Images were taken every 10 seconds unless stated otherwise with an exposure of 100-400 milli-seconds. To reduce the photo damage the light exposure was kept to a minimum by increasing the camera sensitivity via using a camera pixel binning of 8x8. The images were saved as .tif files on a computer dedicated for this purpose only, analysis of data was done post experiment completely offline.

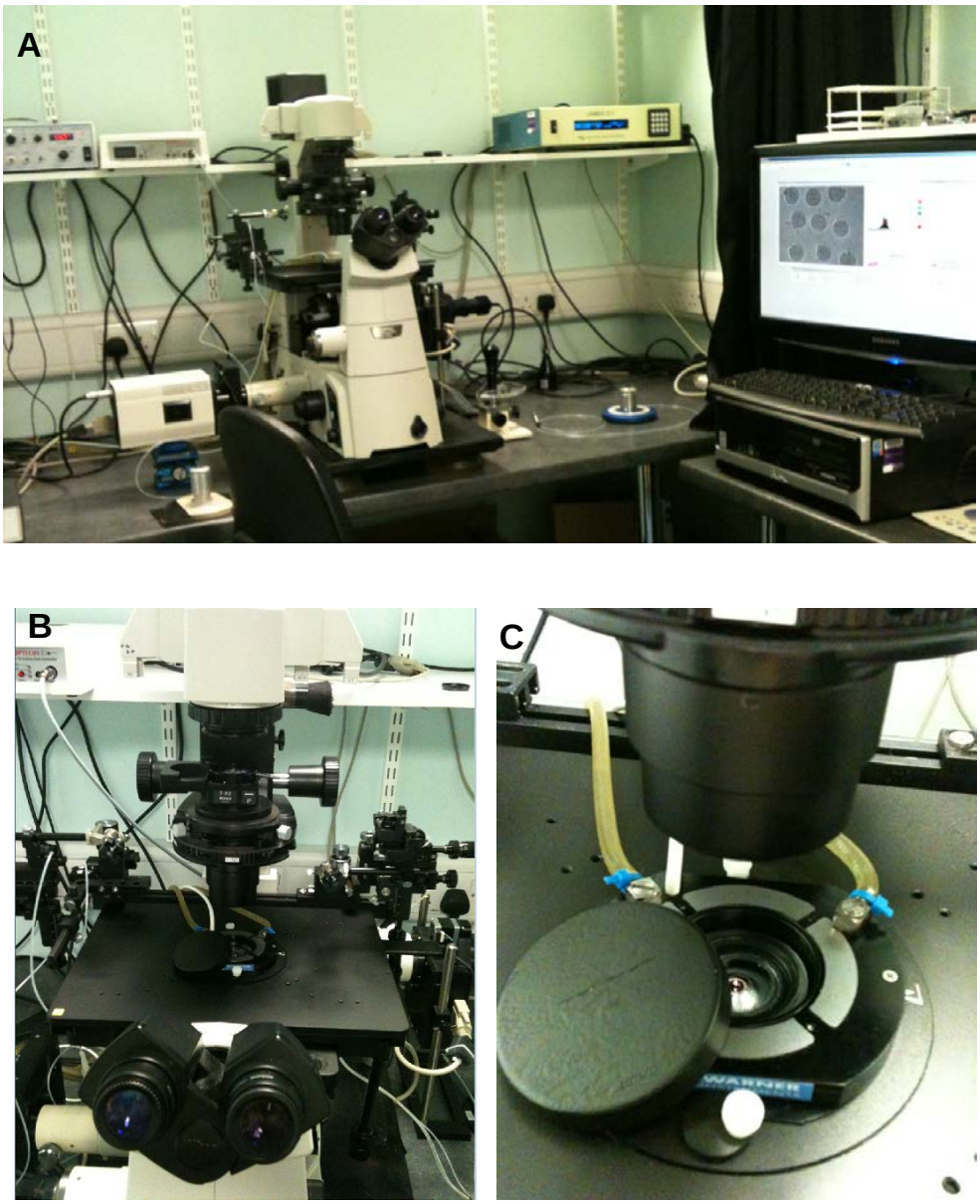

Figure 2.6 The fluorescence imaging system

A, The fluorescence imaging system (inverted microscope, Nikon ECLIPSE Ti-Japan) and the recording of experiments. **B, C** Closer look of the heated stage containing the imaging dish.

2.15 Data and statistical analysis

Data collection and initial analysis is done by specialised software dependent upon the system used for imaging.

For the epifluorescent system the recordings were analysed using the acquisition software Image J and its multiple processing plugins, Image j has the ability to read .tif files which the data is stored as. The .tif files were pulled up and with the use of the multi-measure plugin the images were analysed as stacks, the software would calculate the pixel value yielding fluorescence intensities. Regions of interest (ROI) are drawn around the eggs with an additional area from a free space which would be used as the background fluorescence, this background fluorescence will be later subtracted from the fluorescence value of each egg to give a corrected fluorescence value for individual eggs. The trace representing Ca^{2+} changes in the egg is plotted against time as normalised values, to normalise fluorescence the background corrected fluorescence (F) was divided by the basal value (F0) giving F/F0.

Various files from different imaging systems are imported finally into Sigma Plot 12 Software (Systat Software inc.) for further analysis. This software allows automated creation of graphs through its various tab options, the large data work sheets accommodate any size of data sets. Statistical analysis is done by this software as well, as it provides descriptive statistics for the worksheet columns, it runs paired and unpaired t-tests between worksheet columns and error bars for graphs of column means or standard deviation.

Chapter 3

A Mouse Model of Male Infertility and Rescue of Failed Fertilisation by PLCζ Protein.

3 A Mouse Model of Male Infertility and Rescue of Failed Fertilisation by PLCζ Protein.

3.1 Introduction

The intracytoplasmic sperm injection also known as ICSI is a widely accepted modality of infertility treatment for couples attending the infertility clinics , when compared to alternative options of treatment it has a superior rates of success specially in male factor causes of infertility (Palermo et al., 1992, Johnson et al., 2013). Even with such a treatment that involves injecting a sperm into the egg, failure rates are still documented which can reach a rate of 1-5% of the cycles treated, meaning that all eggs collected form a women have failed to show signs of fertilisation (Liu et al., 1995, Yanagida, 2004). The inability of the sperm to activate the egg is considered the main cause of fertilisation failure in these cases and that is due to a lack or deficiency of factors that allow the egg to be released from its arrest during fertilisation leading to lack of signs of egg activation (Javed et al., 2010, Vanden Meerschaut et al., 2013b, Neri et al., 2014). However, it has to be said that these cases are rare to occur as the egg yield during a cycle of ovarian stimulation yields around 10 eggs to be injected in a single setting of treatment (Sunkara et al., 2011). In case of total fertilisation failure the couple have very limited options for treatment, but even with these options overcoming the problem is not always the possible (Yuzpe et al., 2000, Heindryckx et al., 2005).

The availability of many eggs during treatment cycles somewhat overshadows the failure rate of single egg injections. In cases where the apparent cause of infertility was a tubal factor only over 60% of the eggs managed to show signs of activation (Bukulmez et al., 2000) , which suggests that about 1 in every 3 eggs the sperm are unable to initiate the activation process. If one takes into consideration that the very first IVF treatment was done on an egg that has resulted from a natural ovulation cycle. No hormonal stimulation protocol allows for a natural selection of eggs to be used for the procedure (Hillier, 2013) then the failure rates seen for individual eggs would be of a concern. Moreover, the call is for the implementation of lighter stimulation protocols in the light of concerns raised over the cost and complexity (Loutradis et al., 2007). More importantly the possibility of long-term

health issues that might rise from the use of such high-order ovarian stimulation protocols (Hillier, 2013). If eggs taken from light ovarian stimulation cycles or even natural cycles were to be used, then if there are a limited number of eggs from such cycles the overall ICSI failure rates would be more alarming (Pelinck et al., 2002). Fertilisation in mammals is a well-studied phenomenon and it has been found that Ca^{2+} is heavily involved in this process. In fact the activation step is dependent on the series of Ca^{2+} changes that occur in a form of a series of transients, in ICSI these changes were observed as well in human and mouse eggs (Sato et al., 1999b).

Due to the scarce source of human eggs and the ethical issues surrounding their use in research, it was mandatory to find an alternative model system that has similar components to human IVF. Mouse eggs have been used frequently to understand the mechanisms behind fertilization and have been used to study human IVF and ICSI as well. For instance they have been used in testing the ability of human sperm in causing egg activation (Araki et al., 2004, Vanden Meerschaut et al., 2013a). Although the frequency of Ca^{2+} oscillations that occur due to injecting a human sperm into a mouse egg is higher when compared to the oscillations seen in human eggs, it is still a reliable method of examining the ability of the sperm in activating the eggs (Yoon et al., 2012, Nikiforaki et al., 2014). It was found that human PLCζ is about 10 times more potent than mouse PLCζ (Yu et al., 2008). It is known that human sperm devoid of PLCζ or with mutations of PLCζ when used to perform ICSI in mouse eggs it has a reduced ability to cause mouse egg activation (Heytens et al., 2009, Kashir et al., 2011, Kashir et al., 2012). That is a reasonable conclusion since PLCζ is the main drive behind egg activation during fertilization. However, a striking result came up in a study by Vanden Meerschaut et al in 2013, where it was found that only 55% of the sperm from fertile men had the ability to cause high frequency Ca^{2+} oscillations that would be consistent with a physiological pattern of oscillations in human eggs (Vanden Meerschaut et al., 2013a, Nikiforaki et al., 2014). The data from previous studies suggests that fertilization failure is a consequence of a lack or diminished Ca^{2+} oscillations and that this is most likely to be caused by an abnormal quality or quantity of PLCζ in sperm. Currently most IVF clinics treating cases of fertilization failure are using a method known as artificial activation to overcome this problem. This includes exposing the eggs post ICSI to

an agent that would cause an artificial rise of intracellular Ca^{2+} such as Ca^{2+} ionophores. An example of these agents is A23187 and ionomycin, it is found that such treatment has been successful in treating some cases of ICSI failure (Kyono et al., 2012). It should be noted that Ca^{2+} ionophores causes a single Ca^{2+} transient that has no resemblance to the sustained series of physiological Ca^{2+} oscillations seen during fertilisation (Rinaudo et al., 1997). The repetitive nature of the Ca^{2+} changes during fertilization seems to be favorable for optimum activation of mammalian eggs and embryo development (Ozil and Swann, 1995, Ducibella et al., 2002). Strontium chloride (Sr^{2+}) is an agent that showed promising results specially in mouse eggs and other mammalian eggs, by its ability to cause Ca^{2+} oscillations that mimic those of natural fertilistaion (Méo et al., 2005, Ma et al., 2005). However, it failed to elicit the same outcome when applied to human eggs (Rogers et al., 2004). PLCζ is yet to be matched in its ability to cause Ca^{2+} oscillations that are indistinguishable from those that take place during fertilization in mammalian eggs including human eggs (Ito et al., 2011, Nomikos et al., 2013, Kashir et al., 2014). It has been previously suggested that PLCζ levels could be an effective indicator of sperm quality (Kashir et al., 2013). Collectively, all of this data has paved the way for potential clinical application of PLCζ to be proposed. It appears that a reduced PLCζ concentration, as well as the discovery of certain point mutations in the gene encoding the protein in human sperm can be correlated with the failure of sperm to activate the egg during fertilization.

3.2 Aims of the chapter

In previous studies it has been proposed that PLCζ can be a potential and promising tool of treating cases of failed fertilisation (Nomikos et al., 2012a). In this section of the project I will be looking into the efficacy of PLCζ in rescuing scenarios of compromised development that we have created in our laboratory. The compromised scenarios were created using heated sperm, the model is explained in the corresponding result chapter. To determine if PLCζ can be used as a therapeutic tool it will have to have the ability to improve rates of development up to the blastocyst stage and these rates should be superior to other current used modalities of artificial activation of eggs. Furthermore, it's also necessary to test that treatment with our tagged recombinant PLCζ protein to show that it would not compromise embryo development in comparison with normal ICSI with sperm that has normal parameters. Currently there is no method of identifying the level of PLCζ protein in an individual sperm before being used for ICSI. Therefore, I wanted to investigate if a dose of PLCζ protein given in cases of ICSI with normal sperm would have any detrimental effects on embryo development up to the blastocyst stage.

3.3 Results

3.3.1 Sperm heat treatment and Ca^{2+} oscillations after ICSI.

A plethora of studies have shown that PLCζ can cause Ca^{2+} oscillations when injected into both human and mouse eggs. Unlike many IVF treatments the single mouse sperm used for ICSI does not necessarily need to be motile or even living. Moreover, previous studies have shown that pretreatment of sperm with heat-drying, sonication and even freeze thawing without any cryopreservation could still lead to normal embryo development although in reduced rates (Wakayama and Yanagimachi, 1998, Kwon et al., 2004, Chao et al., 2012). Data shows that mouse sperm when heated to a certain degree and used for ICSI eggs failed to show sign of activation. However, when eggs injected with the same sperm and parthenogenetically activated using Sr^{2+}, only then embryo development was achieved in rates comparable to that of fresh sperm (Cozzi et al., 2001). PLCζ like many proteins is shown to be heat sensitive (Perry et al., 1999), therefore we created our own mouse model of male infertility. To determine if the duration of heat treatment had any effect on the ability of sperm to cause Ca^{2+} oscillations, mouse sperm post collection was exposed to mild heat treatment at a temperature of $56C^{o}$ for various time points starting for 2.5 minutes, 5 min, 10 min, 20 min and finally for a period of 30 minutes. Figure 3, 1 shows the protocol that we have used for the heat treatment which is followed by a brief sonication to separate the sperm heads from the tails for the ease of use during the procedure.

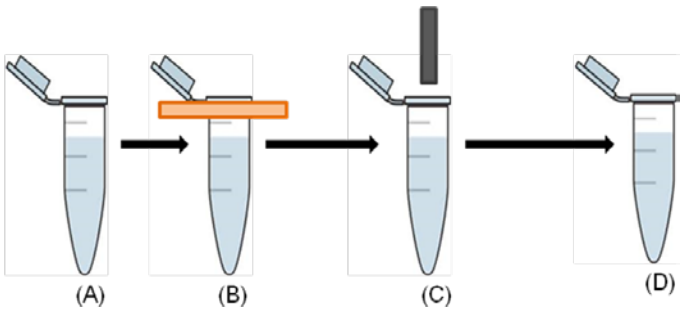

Figure 3.1 Sperm heat treatment protocol.

Sperm after being collected are aliquot as 250µl and stored at -80, prior to use the aliquot is allowed to thaw **(A)**. **(B)** Sperm is place on a floating rack in a water bath heated to $56^{o}C$ and kept for the required duration. **(C)** The sperm after cooling on ice, they are subject to a brief period of sonication 2-4 pulses. **(D)** Sperm post treatment is kept on ice until use for a same day ICSI procedure.

Figure 3, 2 illustrates the Ca^{2+} oscillations recorded after injecting the eggs with (A) fresh sperm, (B) sperm treated for 5 minutes, (C) 20 minutes and finally (D) for 30 minutes.

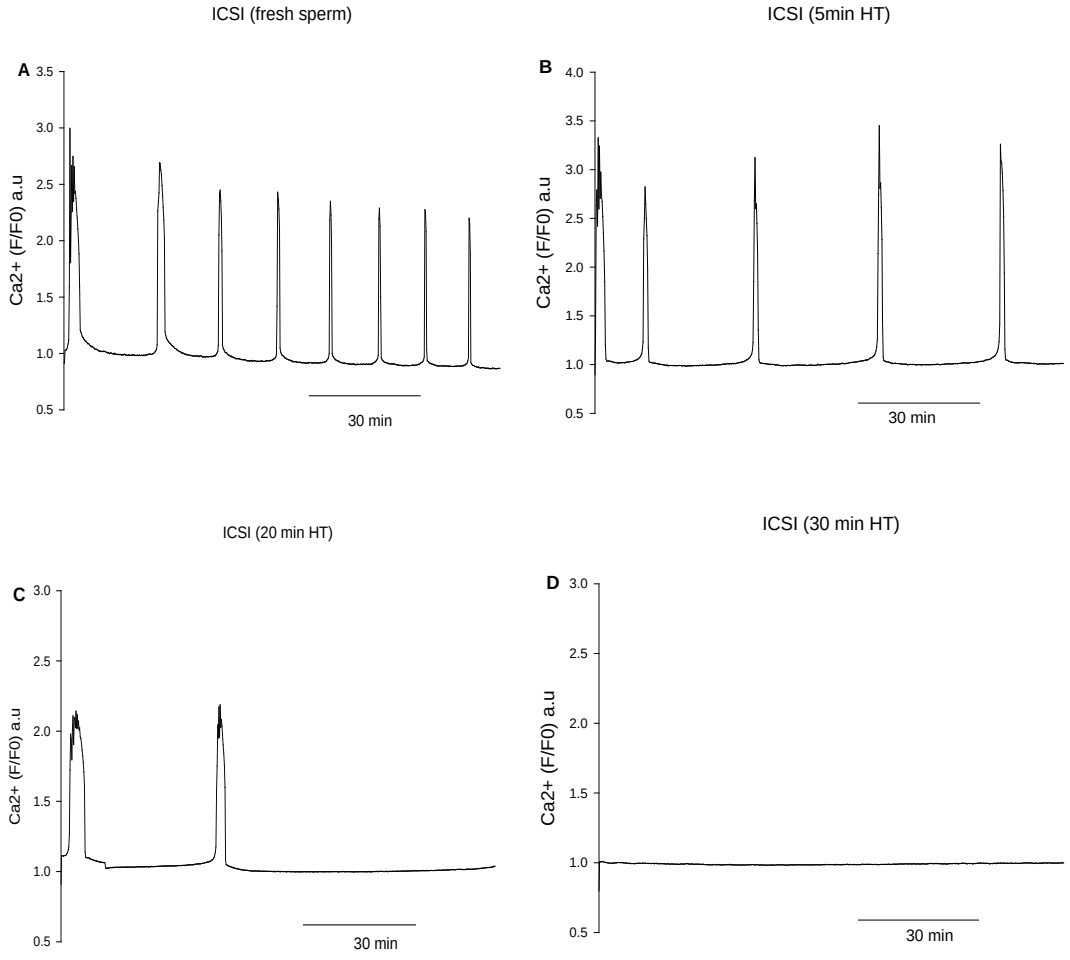

Figure 3.2 Pattern of Ca^{2+} oscillations post ICSI treatment with various heat treatments.

Ca^{2+} oscillations were recorded for the eggs post ICSI with sperm that had **(A)** No heat treatment. **(B)** A representative trace for Ca^{2+} oscillations after heat treatment of sperm for 5 minutes. **(C)** Heat treatment duration of 20 minutes. **(D)** Ca^{2+} spikes for sperm heat treated for 30 minutes. All recordings were continued for 2 hours.

All recording were continued for 2 hours for statistical purposes. In the case of the fresh sperm ICSI we can see a trace of what represents a physiological pattern of Ca^{2+} oscillations. However, as the duration of heat treatment increases the number of Ca^{2+} spikes detected is significantly reduced in parallel over the 2 hour period of recording (Figure 3.3). The findings indicate that even a short duration of 2.5 minutes of heat treatment led to a significant reduction in the number of Ca^{2+} spikes recorded. However, the freeze-thawing process, without the use of any cryopreservants, has not influenced the ability of the sperm injected to cause Ca^{2+} oscillations. The number of Ca^{2+} oscillations detected in the freeze-thawed sperm group was not significantly different from those seen produced by injecting fresh sperm into mouse eggs. Figure 3.3 shows that the numbers of Ca^{2+} spikes are reduced with increasing the duration of heat treatment. There is a point reached where the sperm is no longer able to elicit any Ca^{2+} changes in the form of spikes. That is found to be at the 30 minute time point of heat treatment (Figure 3.2 D). Therefore our data suggests that the sperm's ability to trigger Ca^{2+} oscillations is heavily influenced by the duration of heat treatment at $56^{o}C$, and the longer the treatment the greater the reduction in the number of Ca^{2+} spikes detected.

3.3.2 Sperm heat treatment and the effect on egg activation and embryo development after ICSI.

Previously it has been shown that embryo development is influenced by the surrounding environmental factors. In fact *in vitro* manipulations have been suggested to be a source of epigenetic modifications as well as it could affect the competency of embryo development (Fleming et al., 2004). In a study by Chao et al 2012 it was shown that heat treatment affected the ability of sperm injected during ICSI to cause egg activation and subsequent embryo development in mouse eggs (Chao et al., 2012). However, the study only investigated the effect of various temperature levels and not the duration of exposure to the heat. As it has been shown in the previous section that the sperm heat treatment affected the number of Ca^{2+} spikes recorded after ICSI, it was only logical to investigate whether this reduction would influence the capacity of the heat treated sperm to maintain egg activation and subsequent embryo development.

The eggs that have been used for all sets of our experiments were from the MF1 strain which has a control rate of development post fertilization of around 50% (Ozil and Swann, 1995, Ducibella et al., 2002).

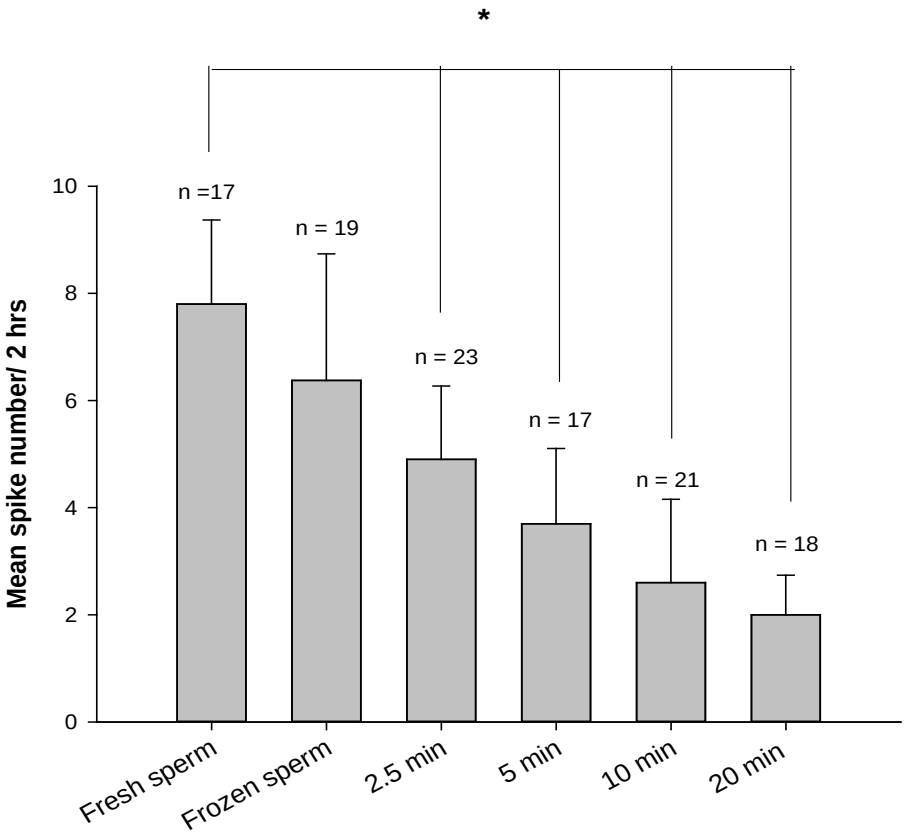

Figure 3.3 A bar graph representing the number of Ca^{2+} oscillations recorded post ICSI with heat treated sperm.

The ICSI procedure was done using sperm that was heat treated for various time points and the resulting number of Ca^{2+} spikes was compared to that of a control using fresh sperm and frozen-thawed sperm.

The rate of egg activation has been taken as the percentage of eggs that develop pronuclei post ICSI in comparison to the number of the surviving eggs post procedure. As expected the fresh sperm managed to activate 100% of the survived eggs after ICSI. A non-significant difference was found between the fresh and the freeze-thawed sperm in terms of pronuclear formation. Table 3.1 shows the percentages of development for each stage and as we can see a small but progressive decrease in rate of activation is detected up to the 10 minute heat inactivation time point. A dramatic decline starting from the 20 minute heat treatment group has been seen which was significant when compared to the fresh sperm activation rate, this significance level was increased for the 30 min group. In a similar manner the rates of blastocyst development have seen a progressive reduction as well. However, the dramatic reduction was seen in an earlier time point (5 min) of heat treatment in comparison to what was detected for the activation stage of development. Between the fresh sperm and the freeze-thawed sperm there was a non-significant difference (P = 0.268), but as soon as the heat treatment was started for 2.5 minutes a significant difference in blastocyst rate development was detected (P = 0.0.0197). Figure 3.4 demonstrates the progressive decline in development for all stages in parallel with the increase in time that the sperm is being heat treated at 56^{o}C. Table 3.1 demonstrates the numbers of embryos at each development stage during *in vitro* culture of embryos. To ensure that there were no confounding factors, each set of experiment was done with a new sperm preparation that was heated for the specific duration required for that experiment and no extension of heating was practiced for the same sperm once used.

From this and the previous set of results we can conclude that the progressive loss of Ca^{2+} oscillations inducing ability of sperm is accompanied by a parallel loss in rates of various development stages after ICSI. Therefore the thermal impact on the sperm function as regards to Ca^{2+} oscillations appears to have been translated into the fertilization rates. Moreover, the effect in some conditions extended to cause fertilization failure, which mimics the clinical scenarios encountered in infertility clinics after some treatment cycles.

Method of activation	No. of surviving eggs (exp.)	No. of eggs activated (PN %)	No. of embryos developing to (%)	
			2-cell	Blastocyst
ICSI with fresh sperm	43 (3)	43 100%	36 83.7%	22 51.2%[a,b,c,d,e,f]
ICSI with frozen sperm	45 (3)	41 91.1%	38 84.4%	12 26.7%[a]
2min 30 sec*	27 (2)	23 85.2%	18 66.7%	6 22.2%[b]
5 minutes*	47 (3)	38 80.9%	28 61.7%	8 17%[c]
10 minutes*	49 (3)	38 77.6%	33 67.3%	3 6.1%[d]
20 minutes*	48 (3)	13 27%	11 22.9%	2 4.1%[e]
30 minutes*	47 (3)	8 17%	1 2%	0[f]

Table 3.1 Developmental rates for mouse embryos after ICSI with heat inactivated sperm heads for various time points.

*Inactive ICSI= Heat treatment at 56^{o}C. a, non-significant difference *P*= 0.268 b, c, d, e, f, represent a significant difference. (P= 0.0197, 0.007, 0.0038, 0.0023, 0.000) respectively.

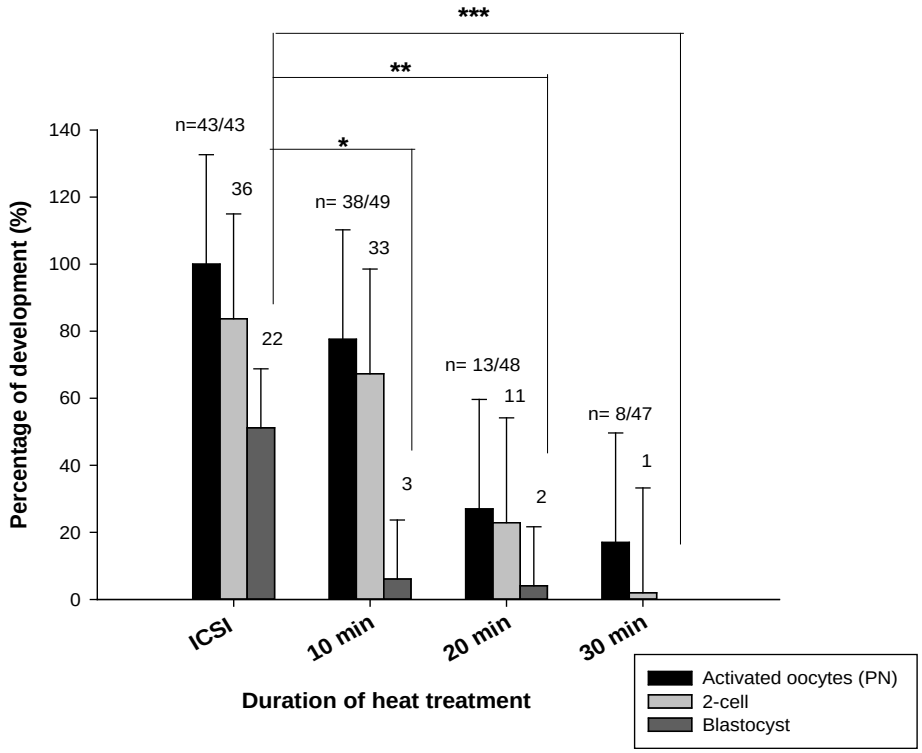

Figure 3.4 Developmental capacity for embryos after ICSI with heat treated sperm for various time points.

Embryo development was followed post ICSI. Each sperm heat treatment time point was evaluated in regard with its ability to support embryo development up to the blastocyst stage. The bar chart illustrates the significant difference of blastocyst rates for each of the heat treatment time points (10, 20, 30min) compared to that of ICSI with fresh sperm. (Student's *t*-test pairwise between the control and each HT group, *P*= 0.0038, 0.0023, and 0.000 respectively) Error bars are SEMs.

3.3.3 Is embryo development after ICSI influenced by the number of Ca^{2+} oscillations?

It has been shown that the number of Ca^{2+} oscillations during parthenogenetic activation of eggs, specifically with the use of electric field pulses, does influence the progression of egg to embryo development (Ducibella et al., 2002). The mechanism of this activation was entirely dependent on influx of Ca^{2+} from the extracellular environment. In light of this result, we wanted to evaluate whether differences in number or pattern of Ca^{2+} oscillations induced in a relatively similar manner to those seen during fertilisation, would have a relative influence on the ability of the embryo to further develop. Furthermore, we wanted to explore whether Ca^{2+} oscillations measured in the same eggs could be seen to have an effect on embryo development progression. MII eggs of MF1 mice were first injected with 30 min heat inactivate sperm as described above. Then they were microinjected with untagged human PLCζ cRNA at a pipette concentration of 0.15µg/µl which was mixed with the Ca^{2+} indicator Rhod-dextran, so Ca^{2+} changes may be recorded. The eggs microinjected would express endogenously the PLCζ protein, for that the initial level of protein would be expected to be at zero and would build up over time. By using this technique, we ensured that the very first Ca^{2+} spike will be recorded. It has been suggested that haploid status of the genetic material is the cause of reduced mouse embryo development rates rather than being caused by parthenogenetic activation (Liu et al., 2002). Therefore, in this experiment it was important that they were subject to ICSI using an inactivated sperm. That would allow for development of diploid embryos that would not have compromised developmental rates. This experiment was done in very small batches (maximum of 14 eggs for each run) as it would include transfer of PLCζ RNA activated eggs into another drop of KSOM that has previously been equilibrated in the incubator. The transfer of eggs is a technically demanding experiment, it was done after spontaneous cessation of Ca^{2+} oscillations that were recorded. The eggs transferred in such a manner such that could be cultured for development and later individual embryos could be related to their corresponding set of oscillations. This was achieved by exposing embryos to time lapse imaging with an image taken every 10 minutes to enable division timing calculation.

The vast majority of eggs arrested at the 2-cell stage were the eggs had a mean number of Ca^{2+} spikes of 21.4 with Std of 10.65 (n = 13), while the eggs reaching the morula stage had a mean number of Ca^{2+} spikes of 15.27/ 4hours and a Std of 1.68

(n = 9) (Table 3.2). We have used a 4 hour period as a cut-off point as a significant number of eggs stopped oscillating before the 5 hour mark 19/35 which was about 54.3% of eggs. The mean values for the Ca^{2+} spikes between the 2-cell stage and the morula stage were significantly different (Figure 3.5), *P* = 0.002. The 2-cell stage arrest was significantly different from the control group where eggs were injected with fresh sperm by ICSI, *P* = 0.006. Moreover, a significant difference was detected in the morula rate between the group that was imaged and the control group (*P* = 0.000). It has to be noted no blastocysts have developed from all imaged experimental runs. Other embryos were arrested at the 4-cell and the 8-cell stage, and no difference was detected when compared to other groups (Figure 3.5).

The results presented indicate that the number of Ca^{2+} oscillations induced via PLCζ protein can influence the ability of the embryo to carry on development at least to the morula stage. However, it has to be mentioned that the overall developmental rates has been compromised in relation to previous parthenogenetic protocols presented in this section. The explanation for such a result could be the amount of light that the embryos have been exposed too. Initially the embryos were imaged for 4 hours to capture the Ca^{2+} oscillation pattern, and then the imaging system was changed as was the light. The long term imaging was done via an ordinary white lamp and an image was snapped every 10 minutes, the total duration was for about 4 days.

A

End stage development of embryo	Mean no. of Ca^{2+} spikes/4hours
2 cell	21.4 (Std 10.56)
4 cell	15.00 (Std 6.69)
8 cell	14.44 (Std 5.43)
Morula	15.27 (Std 1.68)

Table 3.2 (A) Table representing mean Ca^{2+} spikes/4hrs and the corresponding developmental stage at which the embryo arrested.

B

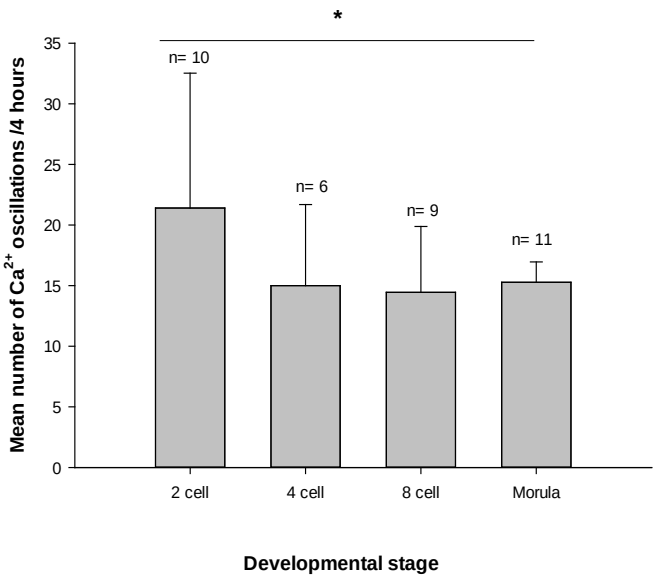

Figure 3.5 (B) End stage development and their mean Ca^{2+} spike number in the imaged group. Error bars are of Std.

A significant difference was detected between the Ca^{2+} spikes for the embryos that arrested at the 2cell stage and the spikes for those arresting at the morula stage. (*P = 0.002).

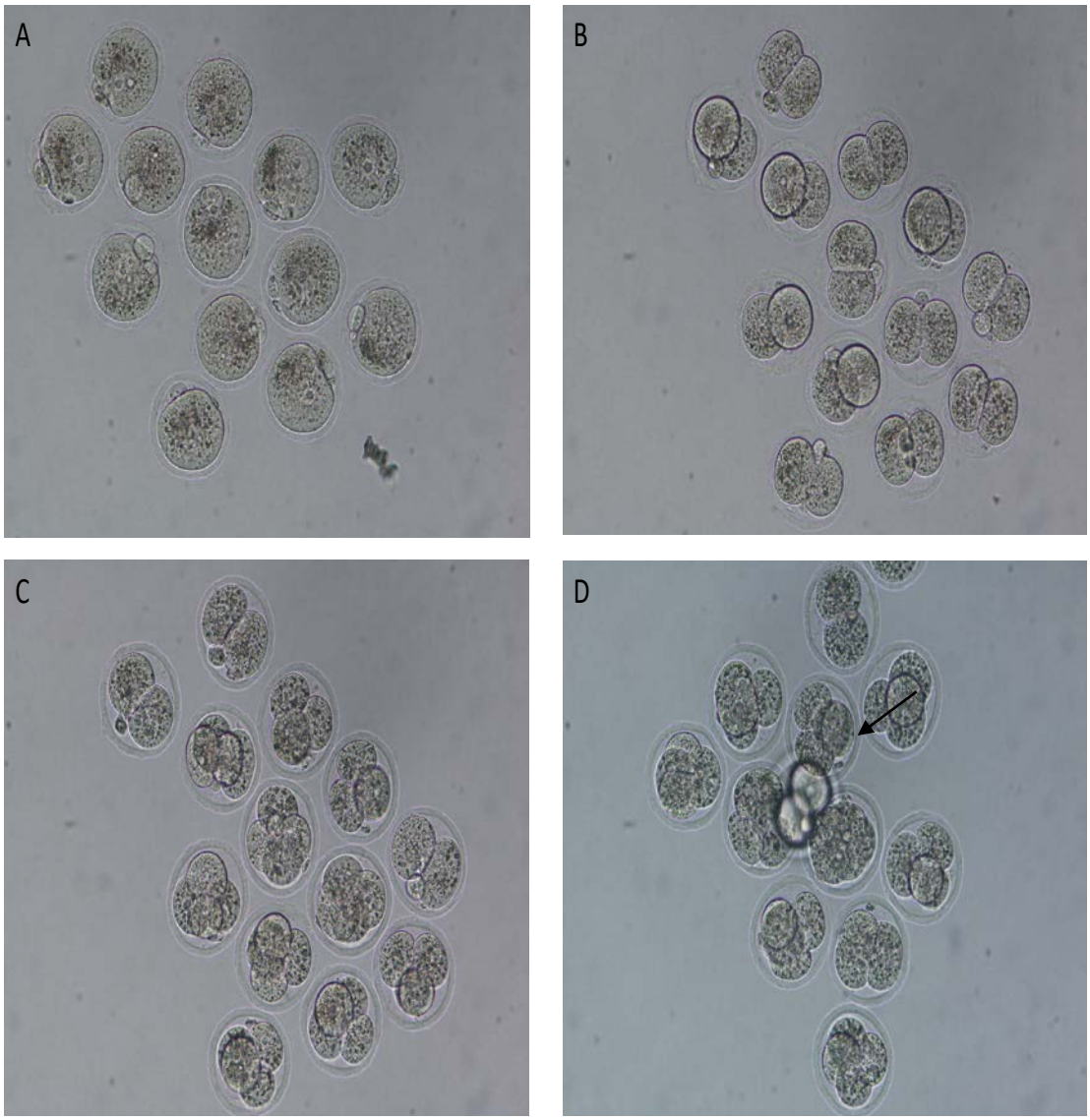

Figure 3.6 DIC images for embryos developing in vitro after imaging Ca^{2+} oscillations.

Images are snap shots for embryos taken during development in the incubator. **(A)** An image for the embryos at the start of imaging showing the PN. **(B)** 24hrs post ICSI and hPLCζ injection the embryos reach the 2cell stage. **(C)** The 4 cell stage, with some embryos arresting at the 2 cell stage. **(D)** At the end of imaging only 1 embryo (arrow) managed to get to the morula stage in this.

3.3.4 Does the pattern of Ca^{2+} oscillations have the ability to affect cell cycle timings?

In 1984 Edwards et al reported that early cleavage of embryos has an impact on the rate of pregnancies (Edwards et al., 1984), as it was shown in his study that the embryos that started to cleave first had a higher success rate of implantation. Since then many studies have looked into the cell cycle timing as a non-invasive tool for determining the quality of embryos and to use as a criteria for choosing superior embryos for transfer (Sakkas et al., 1998, Lonergan et al., 1999, Wong, 2010). In the previous set of results we have shown that the number of Ca^{2+} oscillations is affected by the duration of sperm heat treatment, which subsequently influences the developmental rates negatively. Here we wanted to investigate does this reduction in the number of Ca^{2+} oscillations have any effect on another marker of embryo quality which is the cell division timings. In this set of experiments, ICSI was performed using 4 groups of sperm that was heat treated (2.5 min, 5 min, 10 min and 20 min). The 30 minute heat inactivated sperm was not used as we have previously shown that it does not cause Ca^{2+} oscillations or egg activation. The control groups were 2 sets, first was the ICSI using fresh sperm and the second was the embryos resulting from *in vivo* mating after super-ovulation of female mice. After ICSI was completed the eggs were allowed to recover from the procedure and were incubated and checked after 4hours every half an hour until the pronuclei were formed. Once pronuclei were formed in any of the embryos, they were moved into a culture dish with pre-equilibrated KSOM media. The dishes containing the embryos were placed on the Lumascope stage which was placed within the incubator which was set to 5% CO_2 and 37.5^{o}C. In order to determine the exact timing of each cell division, images were automatically acquired every 5 minutes at a resolution of 20x until about 48hrs post fertilisation. The first cell cycle duration was taken as the time from pronuclear formation until the start of cleavage, while the second cell cycle timing was taken as the time from the completion of the first cell cycle with the formation of two separate daughter cells until the start of the second division. Embryos that did not manage to complete the second cell cycle by dividing into 4 daughter cells were excluded from the analysis.

The analysis was done in a retrospective manner with the use of image-J software, all embryo events were noted and the timing of events as recorded. Figure 3.7 shows a progressive prolongation of the duration taken to complete the first cell cycle in all time points of heat treated sperm. All groups were significantly different from the controls, the 10 minute heat treatment time point was significantly different from the 2.5 minute and 5 minute heat treatment timing. Again the recordings for the second cell cycle timing also revealed the same pattern of prolongation. Time taken to form the 4 daughter cells was increasing in parallel with increasing the duration of heat treatment. All groups were significantly different from the controls. The 10 minute time point showed a more pronounced effect on the second cell timing duration in comparison to the other heat treatment time points (Figure 3.7). Although differences were detected we could not detect any significant differences in between both of the controls in any of the cell cycles. These data suggest that the number of Ca^{2+} oscillations could play a role in influencing the cell cycle timings for embryo development.

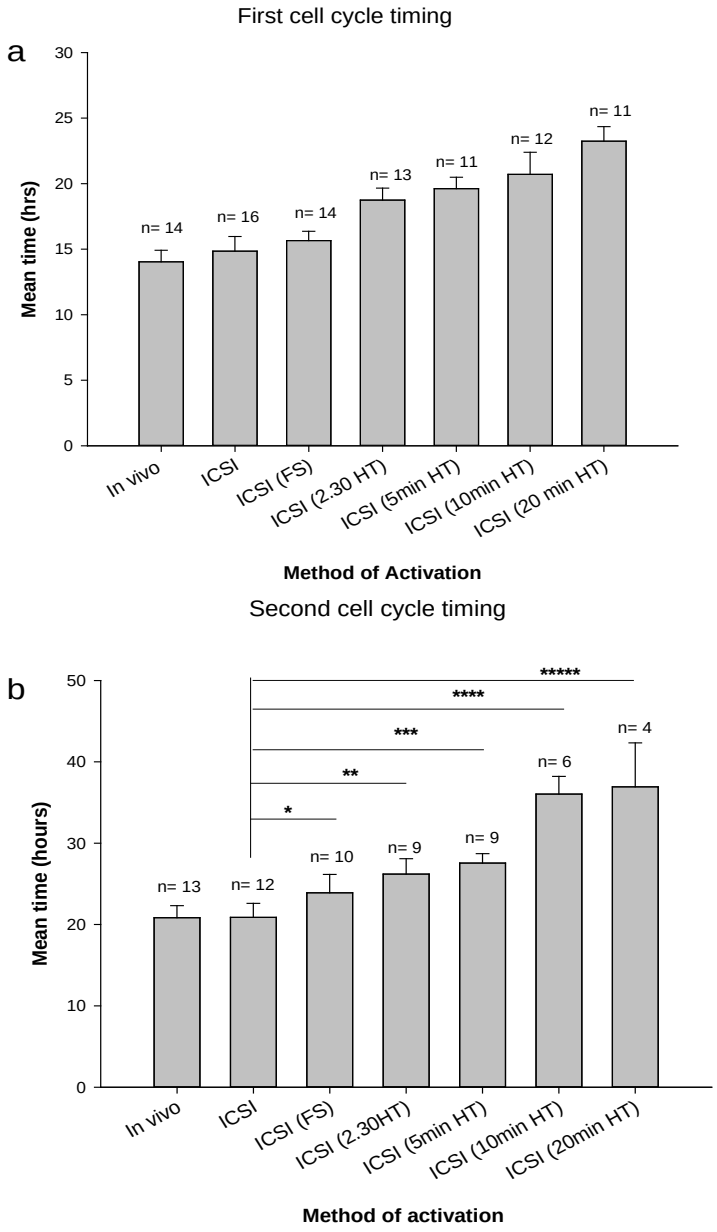

Figure 3.7 Bar graphs representing the timings for the first and second cell cycle division.

(a) The first cell cycle was observed as the period from the pronuclei formation until complete separation of the two daughter cells One way-ANOVA indicates significant differences between the means of the independent groups $P<0.001$. **(b)** The second cell cycle was the time taken after completion of the first cell cycle until full separation of the f daughter cells. The 2nd cell cycle timing showed no significant difference between the in vivo fertilisation and ICSI with fresh sperm. The second cell cycle timing was significantly different between the ICSI with fresh sperm and the other groups.

*P=0.01, **P= 0.008, ***P= 0.0062, ****P= 0.000, *****P= 0.000

3.3.5 Ca^{2+} oscillations are restored with PLCζ following ICSI with sperm which is unable to do so.

Following the introduction of ICSI into the IVF world, increased success rates of fertilization up to 80% and clinical pregnancies detected in 45% of cases treated. Therefore, the need for using donor sperm insemination for couples with severe male factor infertility has been reduced quite dramatically (Palermo et al., 1992). Despite the significant success in rates of fertilization with ICSI, there are cases of total fertilization failure reported as 1-3% of all ICSI treatments see this lack of success (Esfandiari et al., 2005). Following ICSI, if fertilization failure occurs, the most common cause is failure of egg activation (Liu et al., 1995). Other less common causes could include egg spindle defects (Swain and Pool, 2008). A number of reports have showed that these couples have benefited from a combination of ICSI followed by artificial egg activation (Heindryckx et al., 2005, Montag et al., 2012). The most common method currently used is exposing eggs to Ca^{2+} ionophores post ICSI (Nasr-Esfahani et al., 2010). In light of the artificial activation protocols used on human eggs, we wanted to explore and compare the efficacy of PLCζ to other activators in eliciting Ca^{2+} oscillations after ICSI with sperm that is incapable of doing so. Figure 3.8 demonstrates the Ca^{2+} changes recorded after exposing the eggs to an artificial activator subsequent to injecting a sperm heat treated for 30 minutes at 56^{o}C (previously we showed it cannot elicit any Ca^{2+} spikes in eggs). In figure 3.9(A) we can see a single short lived Ca^{2+} rise induced by the action of 5μM of ionomycin which does not resemble the physiological series seen during fertilization. The ionomycin has been added to the media surrounding the egg and washed out after a period of 5 minutes to mimic the protocol used in IVF clinics (Rinaudo et al., 1997). In figure 3,9 (B, C) Ca^{2+} traces are recorded for eggs undergone ICSI with a 30 minute heat inactivated sperm followed by either a Sr^{2+} treatment or an injection of PLCζ. Both traces show a Ca^{2+} change that is similar to the physiological oscillations seen in fertilization. In fact the PLCζ trace is indistinguishable from the physiological trace. The Sr^{2+} media caused a series of Ca^{2+} oscillations (with a mean of 7.91 Ca^{2+} spikes/ 2 hours with a standard deviation of 2.64 (n = 15)) following ICSI using a sperm that has been heat treated for 30 min. However, Sr^{2+} is unable to generate any Ca^{2+} changes in human eggs and since we are aiming to reproduce our results in human eggs we used PLCζ protein for most of the studies. Once mouse eggs were injected with a 30 min heat treated sperm a

subsequent injection with PLCζ was done, the amount of protein used for this rescue was comparable to what has triggered a physiological Ca^{2+} response.

These experiments show that in eggs that have been injected with sperm which lost its ability to elicit Ca^{2+} oscillations, the Ca^{2+} signal was restored by the use of a number of agents such as ionomycin and Sr^{2+} . Both previous agents were added externally to the surrounding media. Signal restoration was achieved by giving a dose of PLCζ protein internally through micro-injection.

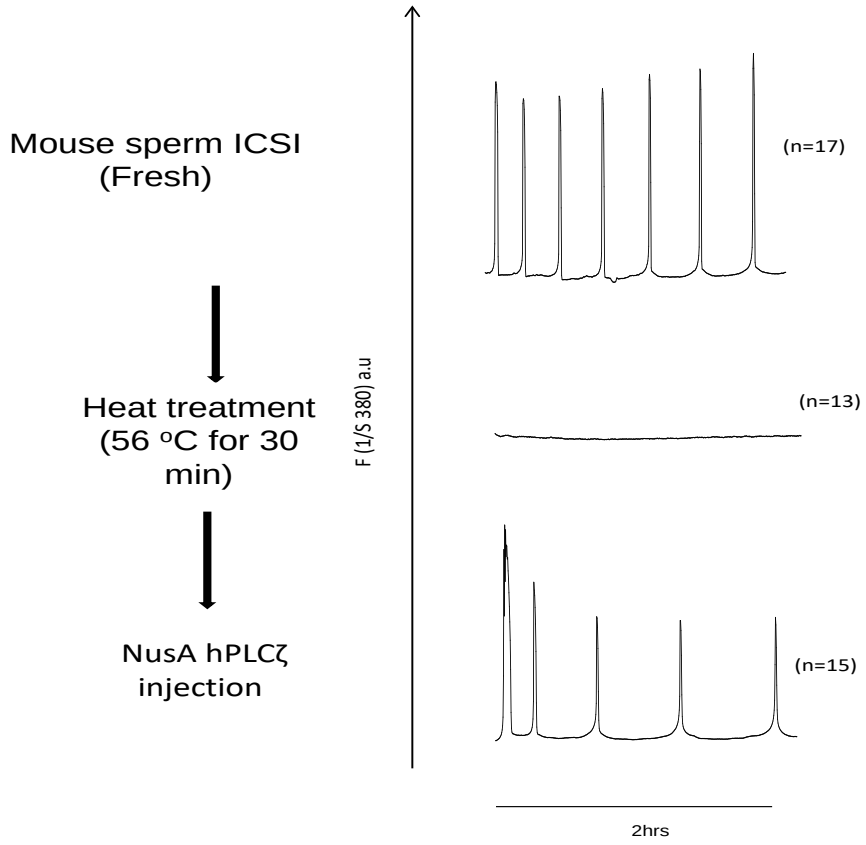

Figure 3.8 Traces are a representation of Ca^{2+} changes following a rescue hPLCζ micro-injection.

A, ICSI with fresh sperm. **B,** ICSI with HT sperm at 56°C for 30 minutes and finally, **C,** restoration of the **Ca^{2+}** oscillations post ICSI with an injection of hPLCζ

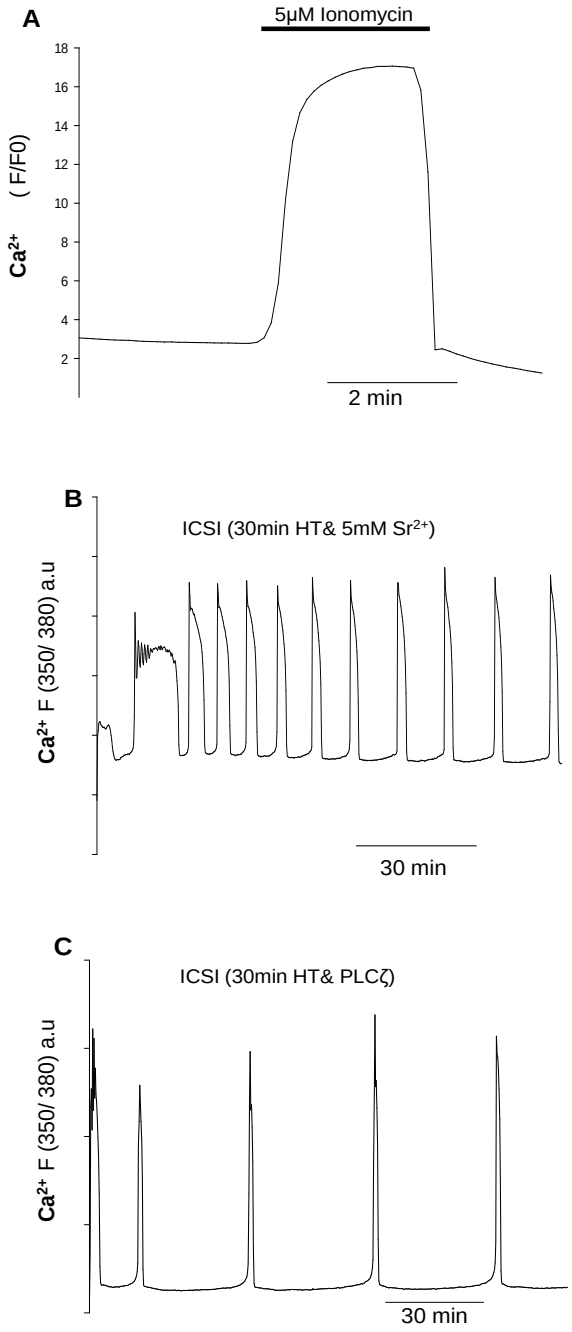

Figure 3.9 Ca^{2+} changes in mouse eggs in response to a number of artificial stimuli.

The use of different artificial stimuli to activate mouse eggs after ICSI with a heat inactivates sperm for 30 minutes. The Ca^{2+} changes were recorded during/following their application. **(A)** Eggs were treated with 5mM ionomycin the trace represents n=21, the bar indicates the start of the application and the wash out of the ionophore. **(B)** Sr^{2+} was applied to the eggs after ICSI, n=12. **(C)** A representative trace for an injection of hPLCζ following ICSI with a sperm subjected to 30min HT.

3.3.6 PLCζ is superior to other artificial activators in rescuing eggs post ICSI with incompetent sperm.

In light of the previous set of results, we wanted to explore the developmental effect of the Ca^{2+} signals that were restored by introducing ionomycin or Sr^{2+} to the media or injecting PLCζ into the eggs that had ICSI with a sperm that was heat treated for 30 minutes. In order to ensure that the rescue was extended to the developmental capacity of the embryos as well, we injected groups of eggs with sperm that was heat treated for 30 minutes which was followed by an artificial activation stimulator. The first group had an exposure to 5µM ionomycin for 5 minutes then washed out in M2 and cultured in KSOM, the second group had the same ICSI treatment then incubated for 4 hours in Ca^{2+} free HKSOM containing 7.5 mM Sr^{2+} which after they were washed out in M2 then cultured as well in KSOM. The last group had ICSI with the same sperm and were subsequently injected with a dose of Nus-A tagged hPLCζ at a pipette concentration of 0.01mg/ml then cultured in KSOM once pronuclei develop and that was the case with the other two groups as well. Following introducing the artificial activators we monitored the activation of eggs which was taken as the development of pronuclei, subsequent stages of development were monitored and documented as well. Control groups were eggs that have been injected with fresh sperm and eggs that had ICSI with frozen-thawed sperm. Figure 3.10 shows that following the ICSI procedure in the case of ionomycin activator the pronuclear development was comparable to other protocols (80%). However, by the blastocyst stage we saw a dramatic reduction to down to only 13.3% of the eggs injected (Figure 3.10). In contrast, when activators that could trigger repetitive Ca^{2+} oscillations were used as opposed to ionomycin that gave a single Ca^{2+} rise, the blastocyst developmental rates were significantly improved, 34.1% and 36.3% for Sr^{2+} and PLCζ respectively. The difference in blastocyst development was not significantly different between Sr^{2+}, hPLCζ and the rates resulting from injecting fresh sperm (P = 0.079). However, ionomycin treatment resulted in a significant reduction in the numbers of blastocysts when compared to ICSI with fresh sperm or rescue with hPLCζ (P = 0.009, 0.0.008 respectively). The use of frozen-thawed sperm in contrast to its similar ability to elicit Ca^{2+} oscillations in mouse eggs, failed to yield blastocysts in rates similar to that of fresh sperm 26.7% compared to 51.2% for fresh sperm (Table 3.3). These results indicate that artificial activators that can trigger Ca^{2+} oscillations such as recombinant hPLCζ can

provide an effective and reliable mean of rescuing eggs that have failed to activate post ICSI.

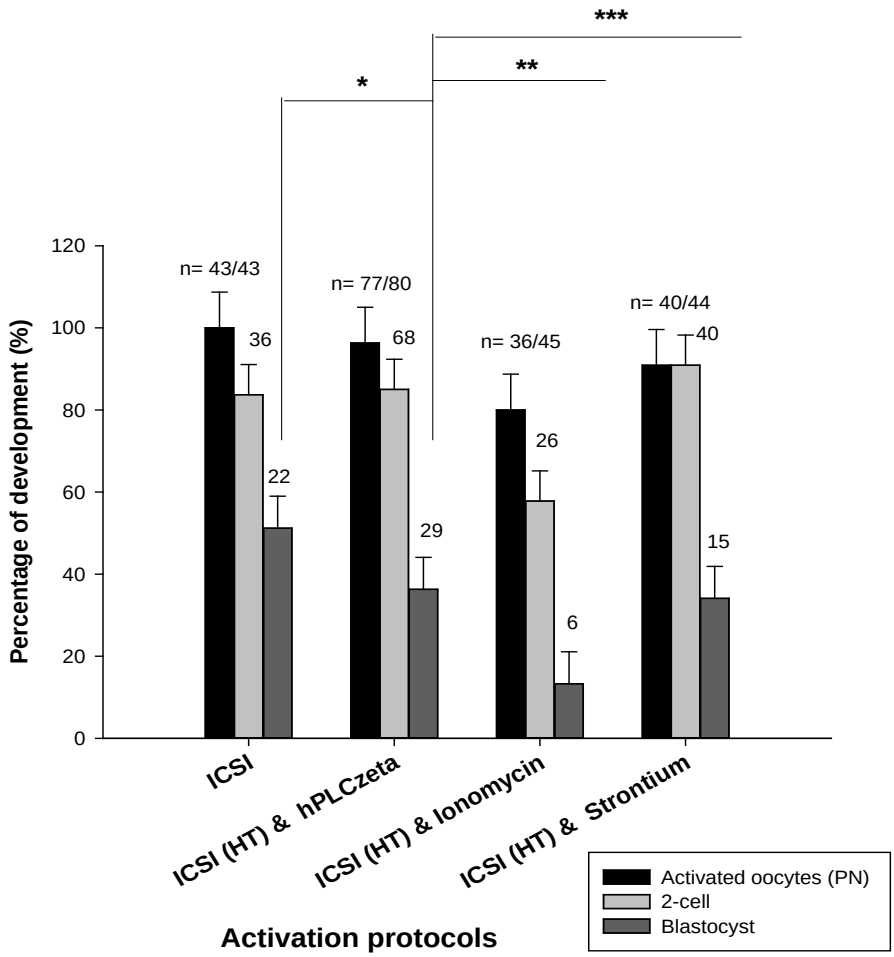

Figure 3.10 Bar chart representing developmental rates for various stages of the mouse embryo following various activation protocols after ICSI with HT sperm for 30 min.

The blastocyst development difference rates were insignificant between fresh ICSI (control) and rescue ICSI with hPLCζ protein injection. While hPLCζ ICSI rescues was superior to egg activation using ionomycin.* P = 0.079, ** P = 0.008, *** P = 0.093.

ICSI method	No. Of surviving eggs (exp.)	No. Of eggs activated (PN)	No. Of embryos developing to (%)	
			2-cell	Blastocyst
ICSI with fresh sperm	43 (3)	43 100%	36 83.7%	22 51.2% [a c]
ICSI with frozen sperm	45 (3)	41 91.1%	38 84.4%	12 26.7%
Inactive ICSI* & 5µM Ionomycin	45 (3)	36 80%	26 57.8%	6 13.3% [a d e]
Inactive ICSI* & 5mM Strontium	44(3)	40 90.9%	40 90.9%	15 34.1% [e]
Inactive ICSI & Nus-A hPLCζ injection	80 (3)	77 96.3%	68 85%	29 36.3% [c d]

Table 3.3 Developmental rates for mouse embryos after various activation protocols post ICSI with HT sperm for 30 min.

Details of developmental stages for the mouse embryos following egg activation protocols, the significant difference detected between the fresh ICSI and the ionomycin activation protocol (*P*= 0.009*),* Fresh ICSI and hPLCζ rescue had no significant difference in blastocyst rates (*P* = 0.079) . d significantly different (*P* = 0.008), e significantly different (*P* =0.059).

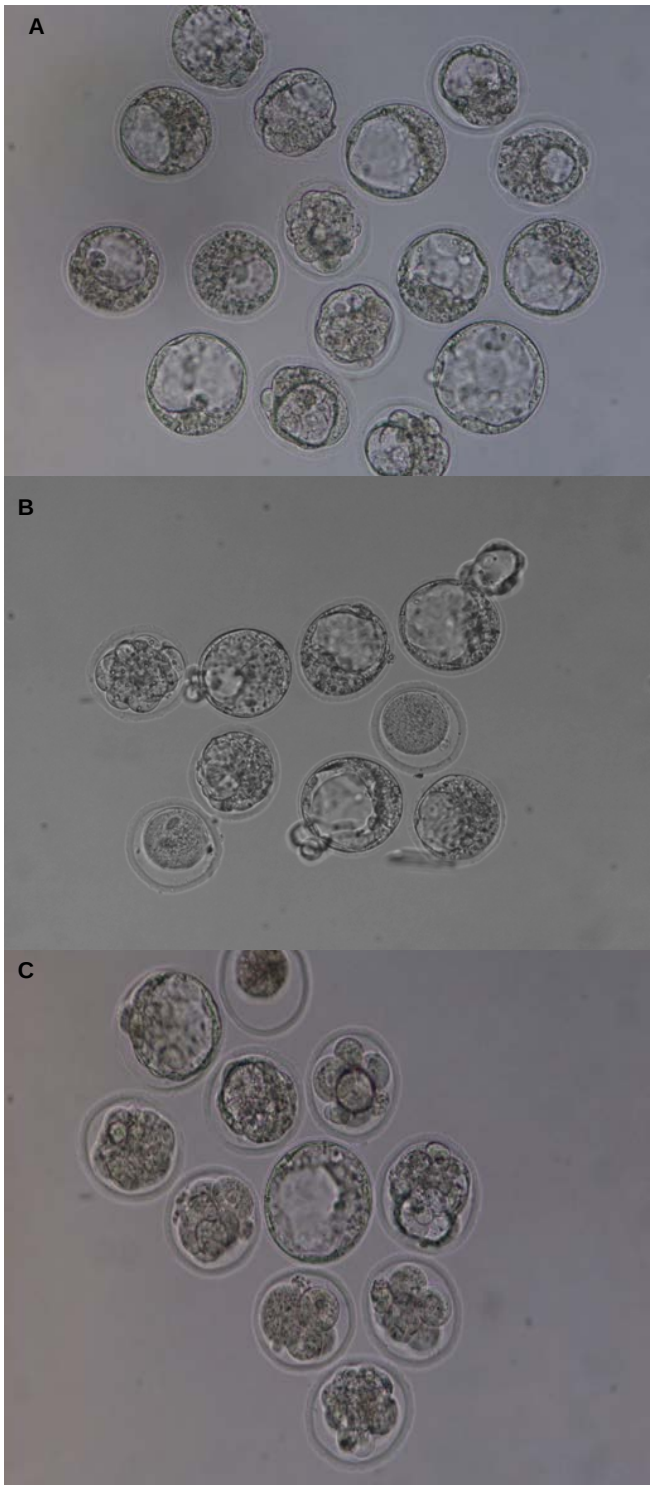

Figure 3.11 Images for in vitro development for various activation protocols.

The DIC images were taken at day5 post ICSI with a HT sperm at 56^{o}C for 30 minutes followed by egg activation using **(A)** hPLCζ protein. **(B)** Sr^{2+}. **(C)** 5µM Ionomycin.

3.3.7 Does a dose of hPLCζ injected into mouse eggs after ICSI with a fresh sperm effect the Ca^{2+} oscillations or subsequent development?

Although success has been reported with artificial activation of eggs after fertilisation failure, rates still have a tendency to be unsatisfactory. This may involve a number of issues such as those related to the time gap between the identification of the failure to fertilise and the application of the artificial activator (Rybouchkin et al., 1997). In normal fertilisation, the activation occurs once the sperm releases the sperm factor into the egg cytoplasm, therefore it would make more sense that artificial activation to be applied during or immediately after the ICSI procedure. However, it is quite difficult to predict cases of egg activation failure, especially in cases where the cause is deficiency in the sperm factor (Kashir et al., 2010). In light of the importance of enhancing the fertilization rates after ICSI and to maximize blastocyst developmental rates, we decided to inject a fresh sperm into an egg that would cause normal activation and then subsequently inject a dose of hPLCζ on top. A double injection technique was followed and the Ca^{2+} oscillations were recorded to identify any deviation from the pattern detected during normal fertilisation. The traces recorded revealed a frequency of Ca^{2+} oscillations that was similar to those seen in ICSI (Figure 3.12 A), or with ICSI using frozen-thawed sperm. It has to be said that we could not identify how much of contribution hPLCζ made to the frequency of Ca^{2+} oscillations between the four groups injected. But we could not detect any significant difference in terms of number of Ca^{2+} spikes recorded in the trace (P = 0.169) (Figure 3.12 B). In previous results we have shown that we can restore development capability of eggs that were injected with heat treated sperm for 30 min. In order to identify if the extra dose of hPLCζ injected into the eggs undergone ICSI with a fresh sperm does not have detrimental impact on embryo development, it was essential to follow development in these embryos. We noted that the extra dose of hPLCζ in addition to what was in the fresh sperm was not detrimental to egg activation (97.3%), 2-cell embryo (78.4%) and blastocyst formation (45.9%). A P value of 0.097 indicated that there was no significant difference between the groups (Figure 3.12 C & Table 3.4). From this set of data we can see that injection of the Ca^{2+} oscillation inducing agent hPLCζ does not appear to impinge on the frequency of Ca^{2+} oscillations, nor does it negatively influence pre-implantation development even when ICSI is performed with what seems to be a normal sperm.

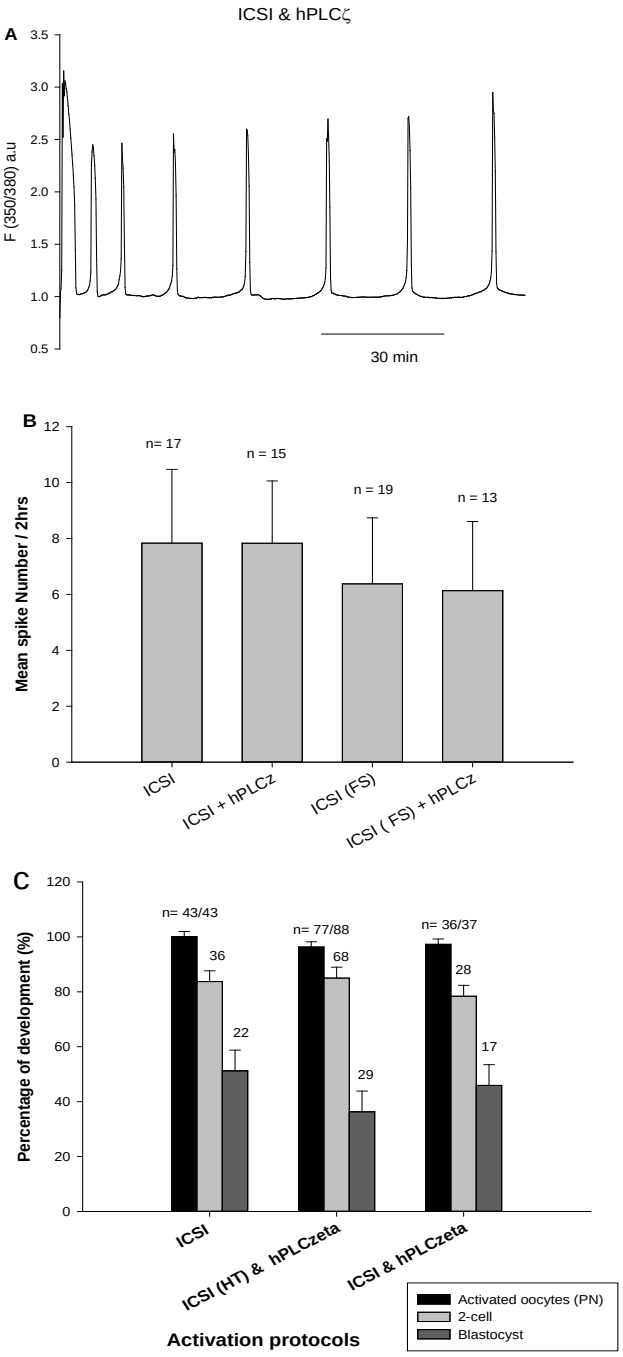

Figure 3.12 Intracellular Ca^{2+} measurements for eggs following ICSI with their corresponding developmental rates.

(A) Representative Ca^{2+} trace for egg post ICSI with fresh sperm followed by a dose of hPLCζ **(B)** The bar chart shows the mean Ca^{2+} spike number post injection with fresh sperm and frozen sperm, fresh sperm and frozen sperm followed by hPLCζ one way ANOVA shows no significant difference between groups (*P* =0.169). **(C)** The bar chart represents developmental rates post ICSI with fresh and HT sperm and followed by hPLCζ injection, one-way ANOVA reveals no significant difference in the blastocyst rates (*P* = 0.097).

ICSI method	No. Of surviving eggs (exp.)	No. Of eggs activated (PN)	No. Of embryos developing to (%)	
			2-cell	Blastocyst
ICSI with fresh sperm	43 (3)	43 100%	36 83.7%	22 51.2% [a]
ICSI with frozen sperm	45 (3)	41 91.1%	38 84.4%	12 26.7% [b]
ICSI fresh sperm & Nus-A hPLCζ injection	37 (3)	36 97.3%	28 78.4%	17 45.9% [a]
ICSI frozen sperm & Nus-A hPLCζ injection	38 (3)	35 92%	29 76.3%	17 44.7% [b]

Table 3.4 Developmental rates for mouse embryos at various stages post ICSI with fresh/frozen sperm followed by hPLCζ injection.

The table represents the rates of development up to the blastocyst stage for mouse embryos that had ICSI with fresh and frozen sperm that was followed by a dose of hPLCζ. The rates were compared to that of ICSI with fresh and frozen sperm alone. The PLCζ injection following fresh sperm ICSI had no effect on developmental rates up the blastocyst stage (*a* no significant difference (P = 0.068)). The frozen sperm ICSI had an improved blastocyst development rate post hPLCζ injection (b, P = 0.024).

3.3.8 The Nus-A tag of hPLCζ, shows no detrimental effects on Ca^{2+} oscillations or subsequent development.

The N-utilising substance A protein (Nus-A) has been used for its ability to keep the partner protein in heterologus expression soluble (Davis et al., 1999). It was found that it can act as a molecular chaperone that inhibits protein aggregation in conditions of stress such as high temperatures and presence of toxic chemicals (Li et al., 2013). As this tag is currently unavoidably micro-injected into eggs along with the hPLCζ protein, it was necessary to determine whether this transcriptional factor would affect the resulting Ca^{2+} oscillations or influence subsequent pre-implantation development in mouse eggs.

Following ICSI with a fresh sperm, Nus-A at a pipette concentration of 0.01mg/ml was microinjected into these eggs and the Ca^{2+} oscillations resulting were recorded and compared to a parallel group of eggs injected with fresh sperm only. Ca^{2+} oscillations were recorded for 2 hours and the mean spike number/2hours were calculated for each group. No significant difference was found between these two groups (P = 0.098). A 10 fold increase in concentration was tested as well, and again no significant difference was detected (P = 0.086). A follow up of development up to the blastocyst stage was done. A control group that was injected with KCL Hepes following ICSI with fresh sperm was also added. All groups were observed in regard to their developmental capacity. In regard with activation rates there was no difference detected between groups. Table 3.5 shows the insignificant rates between groups. Pronuclear formation was 96.5%, 89.4% and 90.7% for KCL Hepes injection, Nus-A (0.1mg/ml) and Nus-A (0.01mg/ml) respectively. However, only 10.6% of the embryos managed to further develop to blastocysts in the Nus-A group of (0.1mg/ml). Whereas 34.9% of the injected eggs developed into blastocysts in the Nus-A (0.01mg/ml) group and the difference was significanct (P = 0.007). Furthermore, when the Nus-A (0.01mg/ml) blastocyst developmental rate was compared to that of KCL Hepes injection no significant difference was found (P = 0.093) and again no statistical difference was detected between the (Nus-A (0.01mg/ml) group and the blastocysts resulting from eggs injected with fresh sperm (P = 0.085). The above data reveal that the Nus-A tag for the PLCζ protein is not detrimental to development in the concentrations that are present with the recombinant hPLCζ protein used.

ICSI method	No. of surviving eggs (exp.)	No. of eggs activated (PN)	No. of embryos developing to (%)	
			2-cell	Blastocyst
ICSI with fresh sperm	43 (3)	43 100%	36 83.7%	22 51.2%[a]
ICSI with fresh sperm and KCL hepes injection	41 (3)	37 96.5%	33 79.6%	18 43.9%[b]
ICSI fresh sperm & Nus-A injection (0.1mg.ml)	38 (3)	33 89%	13 32.5%	4 10.6%[c]
ICSI fresh sperm & Nus-A injection (0.01mg/ml)	36 (3)	33 90.7%	28 78.3%	13 34.9%[a, b, c]

Table 3.5 Nus-A injection effect on the developmental ability of mouse embryos.

The table represents the developmental capacity of mouse embryos post ICSI with fresh sperm and an injection of 2 different concentrations of the Nus-A tag (0.1mg/ml, 0.01mg/ml). The control groups were ICSI with fresh sperm and ICSI with fresh sperm with a KCL-hepes injection. No significant difference between the Nus-A 0.01mg/ml injection post ICSI and the fresh ICSI, ICSI with KCL-hepes, (P = 0.085, 0.093 a, b respectively). A significant difference in the blastocyst development rate was detected between the concentration of Nus-A injections (P = 0.007).

Discussion

Occasionally during IVF treatment cycles including ICSI, cases of total fertilisation failure are reported. Either sperm or egg related causes could be the origin of this failure. However, the most common cause is due to egg activation failure (Nasr-Esfahani et al., 2010, Javed et al., 2010). In the study presented, we have shown that the mouse model of male infertility that we generated had an effect on its ability to induce Ca^{2+} oscillations in mouse eggs. The model was generated via exposing the sperm to mild heat treatment of 56^{o}C. This level of heat treatment has been shown not to have any influence on the genetic material such as causing DNA damage (Perry et al., 1999). Not only DNA is not affected, no detrimental effects on embryo development were found in cases where independent activation protocols were applied to the eggs. Though the importance of Ca^{2+} oscillations for egg activation is undisputed, there is still some debate about the identity of the cause behind the trigger of these changes. However, the most widely accepted view is that the sperm specific PLCζ, which is released into the eggs during fertilization triggers the series of Ca^{2+} oscillations via IP_3 Ca^{2+} induced release (Saunders et al., 2002). In addition multiple independent research teams have established that this protein has the ability to trigger the release of Ca^{2+} in mammalian eggs (Kurokawa et al., 2004, Miyazaki and Ito, 2006, Nomikos et al., 2014). This factor, initially described as the SOAF (sperm oocyte activating factor) has been previously identified to be within the sperm perinuclear theca, it has been shown that this protein is heat labile in earlier reports of mouse ICSI (Perry et al., 1999).

Our data is in agreement with all these previous findings. The results presented have shown that the triggering ability for Ca^{2+} oscillations after ICSI is affected when sperm are exposed to mild temperatures (56^{o}C) for 30 minutes. Furthermore, not only did the heat treatment influenced the activity of PLCζ, but the process of freeze-thawing of sperm with no cryopreservation has been shown to negatively affect the protein activity as well and this was the findings in a study by (Kashir et al., 2011). The freeze thawing cycle has led to a reduced cell numbers at different developmental rates up to the blastocyst stage in comparison to those seen when fresh sperm was used, and yet, no statistical significance was detected. The partial loss of protein activity could be related to either an inactivation process or due to a reduction in the quantity of protein within the sperm during the course of freeze-thawing procedure. Whatever the cause of this reduction in activity, our current

concern was to focus on the consequences of this activity reduction if the sperm was to be used to fertilise eggs. In this sense, the slight reduction in activity with frozen sperm has been beneficial for this project as it has provided a means to mimic clinical scenarios presented to the IVF clinics where there is only a very slight loss of sperm factor activity.

The MF1 mice used for our experiments have a known rate of blastocyst development of around 50% *in vitro.* This is unlike the F1 hybrid mice which have a higher rate of blastocyst developmental rates exceeding 90%. This lower rate of blastocyst development was again useful in our studies, since this level mirrors the rates that are reported in human eggs (Loutradi et al., 2006). When sperm was treated at 56^{o}C for different time points it led to a stepwise reduction in the number of Ca^{2+} oscillations resulting when eggs were injected, enabling us to create a range of affects seen later in embryo development. The increasing duration of heat treatment that was applied to batches of sperm had parallel affects which were seen on activation rates and further embryo development up to the blastocyst stage. In fact the complete loss of Ca^{2+} oscillations triggering ability of sperm, when heat treated for 30 minutes, saw an exact match of 0% blastocyst developmental rate after ICSI. A 50% reduction in the blastocyst developmental rate was seen when the shortest duration of heat treatment was applied (2.5 min), however that again was seen in the corresponding number of Ca^{2+} oscillations for that group. To sum up, it is evident that a significant reduction of Ca^{2+} oscillations is accompanied by a significant reduction in blastocyst developmental rates. That is in agreement with previous studies suggesting that each stage/event of embryo development is influenced by the number of Ca^{2+} oscillations (Ducibella et al., 2002).

It is noteworthy that the Ca^{2+} oscillations induced in our study are of a physiological nature unlike the study mentioned where the Ca^{2+} transients were induced via electro-permeabilisation, hence the lack of the signature first Ca^{2+} spike which is specific to fertilisation. An interesting finding was in a study by F. Vanden Meerschaut et al 2013, where human sperm that failed to fertilise eggs after ICSI were injected into mouse eggs. In general a very low frequency or defective Ca^{2+} oscillatory pattern was the case (Vanden Meerschaut et al., 2013a). These findings have the strength to reveal that the Ca^{2+} oscillatory pattern has the ability to predict egg activation failure and subsequent diminished rates of further embryo development. When a clear correlation was made between fertilization failure and

the lack or deficiency in Ca^{2+} oscillations, a treatment option was to consider an artificially induced cytosolic Ca^{2+} change via Ca^{2+} ionophores (Neri et al., 2014). There are cases of success that have been reported using this method of treatment after a history of complete fertilization failure, not just clinical pregnancies were documented but live births have been recorded (Eldar-Geva et al., 2003, Murase et al., 2004, Yanagida et al., 2008). Although these chemicals allow intracellular Ca^{2+} release, they only cause a single large transient. In order to mimic fertilization these will have to be applied multiple times or in combination protocols with Ca^{2+} injection (Heytens et al., 2008, Nasr-Esfahani et al., 2010). However, concerns are continuously raised over the use of such chemical agents as they could possibly be detrimental to the embryo if not immediately, then later during development. The only agent reported that can cause Ca^{2+} oscillations, is Sr^{2+} and it has a variable egg activation success rates reported depending on the species used (Yanagida et al., 2008) with low success reported on human egg activation (Chen et al., 2010a). Ionomycin has been used in this presented study as a fertilization failure rescuer where ICSI with heat treated sperm for 30 minutes was used. Activation rates were successfully restored to rates of 80% yet still was significantly different to that of ICSI with fresh sperm (100%). When Sr^{2+} was used as an artificial activator it was superior to ionomycin in both activating eggs and the blastocyst development rate (90.9%, 34.1% respectively). When cRNA encoding the full length of PLCζ protein was injected it was found that induced Ca^{2+} oscillations equivalent to that triggered by fresh sperm (Cox et al., 2002, Saunders et al., 2002). These oscillatory patterns induced had the ability to support embryo development up to the blastocyst stage (Rogers et al., 2004). However, injecting PLCζ cRNA will prove to be clinically unacceptable, it is genetic material and human reverse polymerase could convert it into cDNA which subsequently be a part of the human genome (Spadafora, 2004). Furthermore, Yu et al (2008) found that activation of eggs can be induced by a wide range of PLCζ concentrations but for successful preimplantaion development it was not the case. In this previous study it was shown that injecting high concentrations of PLCζ leads to a high frequency of an oscillatory Ca^{2+} patterns and this leads to cleavage arrest. It appears that there is a window of PLCζ concentrations that would give preimplantaion developmental rates similar to that of normal fertilization (Yu et al., 2008). However, PLCζ recombinant protein has been successful in activating human eggs that were subject to ICSI and failed to fertilise (Yoon et al., 2012). The previous results have set a stage for the use of recombinant PLCζ protein to be

used as a therapeutic agent for fertilization failure. However, there have been no reports of its comparison to other artificial activation protocols and agents.

In my results I have shown that hPLCζ protein when used for activating eggs that were injected with a 30 minute heat inactivated sperm, the success of rescue was almost as that for ICSI with fresh sperm (96.3% vs 100%). It is noteworthy that Sr^{2+} showed no difference to PLCζ's ability in activating eggs that were otherwise destined for failure of fertilization. But the questionable applicability and efficiency of Sr^{2+} on human eggs renders PLCζ in a stronger position. However, the effects of Sr^{2+} emphasize that the rescue of activation is entirely due to Ca^{2+} signals. In this protocol of rescue, PLCζ protein was microinjected into the egg using a high pressure injection system after the ICSI procedure. This method of injecting the eggs twice has been used at least in another setting to inject Ca^{2+} after injecting a sperm via ICSI as a part of a rescue protocol (Vanden Meerschaut et al., 2013b). It is more likely that human eggs would manage to endure a second injection more that mouse eggs as they are more robost. For example, in mouse ICSI a piezo device is to be used to enhance survival rates form 50% to 90% whereas human ICSI needs no such special equipment to obtain high survival rates (Swann, 1990, Yoshida and Perry, 2007). Therefore, it would not be expected that the second injection would be detrimental to pre-implantation development in human eggs. The concern over the use of artificial material in activating eggs would not be of much a concern, due to the fact the protein is an endogenous protein in sperm (Perry et al., 1999). Moreover, the pattern of Ca^{2+} changes induced by PLCζ protein is identical to that seen at fertilisation.

As shown in a study by Vanden Meerschaut in 2013, sperm of fertile men who were considered as controls showed that only above 50% of their sperms produced the pattern of oscillation that was considered sufficient for successful fertilization (Vanden Meerschaut et al., 2013a). Moreover, immuno-cytohistochemistry studies have shown that the level of PLCζ protein in human sperm could be quite variable within the same sample of ejaculate (Kashir et al., 2013). The ICSI procedure includes selection of sperm to be used for the procedure, the selection criteria include morphology and motility (Antinori et al., 2008). To assess the ability of any given sperm to trigger the necessary Ca^{2+} oscillations for successful fertilization, currently it is unfeasible for the embryologist to recognize. Therefore, it is a possibility that a number of eggs will get some PLCζ protein from sperm on top of

the dose from the recombinant PLCζ protein injected. From this perspective we investigated this scenario. Eggs that had ICSI with a fresh sperm had a second injection of recombinant PLCζ protein in a dose that is sufficient to cause a physiological pattern of Ca^{2+} oscillations. We also applied the same protocol to eggs that have been injected with a freeze-thawed sperm, which causes a non-significant reduction in Ca^{2+} oscillations when compared to fresh sperm. In both scenarios we found that this extra dose of PLCζ had no detrimental effects neither on activation nor blastocyst development rates. This might seem surprising in light of previous studies, which revealed that high frequency oscillations due to protein over-expression led to poor preimplantation development (Yu et al., 2008). However, this injection is still within limits of the window identified for tolerance which involves about a fourfold difference in PLCζ concentrations (Yu et al., 2008). My results are also in agreement with studies showing that the fusion of two sperms with an egg as opposed to a single sperm showed only a very small change in the frequency of Ca^{2+} oscillations (Faure et al., 1999). The presented data suggests that the injection of PLCζ after ICSI as a protocol for rescue of activation failure is a viable alternative to those currently used in the IVF clinics. Moreover, it could be applied without causing any detrimental effects on embryo preimplantaion development.

Chapter 4

A Comparison Of Injecting PLCζ Protein Into Mouse Eggs And Human Eggs And The Search For An Alternative Method Of Injection

4 A comparison of injecting PLCζ protein into mouse eggs and human eggs and the search for an alternative method of injection.

4.1 Introduction

The pattern of Ca^{2+}oscillations induced by an injection of hPLCζ into mouse eggs was indistinguishable from those induced by fertilisation. The Ca^{2+} oscillations were able to support embryo development up to the blastocyst stage (Saunders et al., 2002). Yu et al provided further support by injecting hPLCζ cRNA into mouse eggs, and he showed that the best blastocyst developmental quality and quantity fell within a specific range of the expressed protein (Yu et al., 2008). Rogers et al published the first data showing the ability of PLCζ cRNA to induce long lasting series of Ca^{2+} oscillations in aged human eggs. The groups work was the first to show that PLCζ protein was able to trigger Ca^{2+} signal that supported development to the blastocyst stage in human eggs that have failed a fertilisation procedure (Rogers et al., 2004). My work presented in chapter 3 and published recently, compared the developmental capacity of mouse embryos activated parthenogentically via different means. The study revealed that hPLCζ protein injection had a comparable rate of blastocyst developmenta to that of fertilisation and was superior to other methods of activation (Sanusi et al., 2015).

IVF programmes are currently looking into ways to overcome potential fertilisation failure that has been documented in some cases (Palermo et al., 1992). Since the ICSI technique has been introduced, some causes of male infertility problems have been treated. However, not all cases have been successful (Yoon et al., 2012). The literature has a plethora of studies highlighting the potential of assisted activation protocols in overcoming egg activation failure post IVF. The methodology basically is dependent on creating a single or multiple Ca^{2+} transients during the IVF procedure, by the use of Sr^{2+} treatment or by other chemical agents as Ca^{2+} ionophore (Borges Jr et al., 2009a, Taylor et al., 2010, Chen et al., 2010a). The concept of assisting the process of fertilisation by the use of chemical agents that essentially trigger Ca^{2+} signalling within the egg, has been questioned in terms of their safety (Nasr-Esfahani et al., 2010). Sr^{2+} was the only agent that managed to trigger Ca^{2+} transients similar to that caused by fertilisation but this response was only shown in mouse eggs. Sr^{2+} has never been found to cause Ca^{2+} oscillations in

human eggs. The successful demonstration of Ca^{2+} oscillation triggered by the use of Sr^{2+} has been limited to rodent models. In humans eggs Sr^{2+} application has only been reported as case reports of successful human egg activation and no traces representing the Ca^{2+} oscillations have been shown (Nasr-Esfahani et al., 2010). Moreover, there have been two reports linking repeated failed fertilisation after ICSI to a maternally inherited point mutation in hPLCζ (Heytens et al., 2009). Further support has been provided in a study by Nomikos et al 2011 when injection of a cRNA encoding the point mutation failed to induce a series of Ca^{2+} oscillations in mouse eggs (Nomikos et al., 2011a). It is only fair to mention other proposed sperm factors that were put forward as potential candidates for rescuing egg activation failure. Another sperm protein known as PAWP (post-acrosomal WW domain-binding protein) was described by Arabi M in 2014 as being a candidate that could fulfil the requirements for being an egg activator during fertilisation (Aarabi et al., 2014a). However, a series of studies challenged that proposal and it seemed that the sperm PAWP hypothesis has not stood the test of time (Nomikos et al., 2015, Escoffier et al., 2016). For those reasons, it would appear that the only available egg activator is PLCζ.

Injecting cRNA encoding PLCζ into human eggs would face legal issues since cRNA is genetic material. The other problem that would encounter such a protocol is the inability to identify the precise translation capability of the injected cRNA into human eggs. For both mentioned reasons the use of PLCζ cRNA in infertility clinics is highly unlikely. For that, research has led on to a search for a more natural form of egg activation, and this led to idea of using recombinant PLCζ protein. While the purification and stability of the synthesized protein is still under scrutiny, the protein has been injected into human eggs that have previously failed to show signs of fertilisation post ICSI and as a result they developed 2PN (when they were injected with 1µ/µl of PLCζ protein) (Yoon et al., 2012). This potential therapeutic molecule has high chances of being used with success within infertility clinics. Studies so far have been focusing on the variations in PLCζ between different species in terms of their ability to cause Ca^{2+} oscillations (Cox et al., 2002, Coward et al., 2005). The functionality of the sperm specific phospholipase as an enzyme has attracted attention as well (Kouchi et al., 2005, Nomikos et al., 2005). Research into egg sensitivity of different species when injected with other species PLCζ was another aspect of interest (Rogers et al., 2004). All previous studies investigated the effect of PLCζ on various species done after a procedure that utilises a high pressure

injection system. Despite the attention that PLCζ has attracted, there were little or no studies investigating the differences in settings for PLCζ injection that exist between scientific laboratories and that of infertility clinics who almost exclusively use low pressure injection systems. The only low pressure systems used in laboratories use an oil drop to separate the culture media from the injection solution. This results in a drop of oil being injected into the egg, which is probably not acceptable in a human egg in an IVF clinic.

4.2 Aims

The aim of this chapter is twofold, firstly to investigate further the effectiveness of injecting Nus-A tagged hPLCζ recombinant protein into human eggs that have undergone an IVF procedure and failed to fertilise. Moreover, to observe the triggered series of Ca^{2+} oscillations in human eggs and compare them to those resulting from the same dose injected into mouse eggs. That would possibly allow for a dose calculation that would be suitable to trigger an appropriate Ca^{2+} response in human eggs. The aim was to elicit a Ca^{2+} response that has the ability to trigger egg activation and support embryo development afterwards. Secondly, I also investigated an alternative method to a double injection of eggs if PLCζ is to be applied after ICSI. To my knowledge I also carried out the first attempts to inject the Nus-A tagged hPLCζ protein simultaneously with sperm during ICSI. In this chapter, I also succeeded in showing that the protein is stable after being freeze dried. This would benefit the supply and transport of this material to IVF clinics as opposed to its original liquefied state. This method of transportation and storage would likely reduce the cost of protein use. The experiments presented here show a systematic approach, by testing various mediums and injection pipettes, which could potentially be used on a regular basis in infertility clinics. The human eggs that were used in this part of the study were donated kindly by patients being treated at the infertility clinic in Neath Port Talbot and Wales infertility institute at Cardiff. The project was approved by the UK Human Fertilisation and Embryology Authority (HFEA) (R0161).

4.3 Results

4.3.1 Human PLCζ recombinant protein induces long lasting Ca^{2+} oscillations in mouse eggs and human eggs that have failed to fertilise post IVF.

Rogers NT et al in 2004 showed that aged human eggs that have failed to fertilise during an IVF procedure including ICSI, have responded to an injection of PLCζ cRNA when various pipette concentrations were used. The range of PLCζ RNA concentration was (0.1µg/ml-1mg/ml) and all triggered repetitive Ca^{2+} oscillations with differences detected in frequency. Higher concentration of cRNA tended to induce a higher oscillation frequency (Rogers et al., 2004). Therefore, in the upcoming set of experiments, Nus-A tagged recombinant hPLCζ protein has been used as an alternative to mimic the expected scenario in clinical settings. Human eggs that have failed an IVF procedure ICSI either IVF or ICSI (day 1 post IVF) have been used in this set of experiments. These human eggs were loaded with an AM dye (Cal-520) for a duration of 45 minutes at 37^{o}C and allowed to recover for about 10 minutes before being injected with a dose of PLCζ protein. Cal-520 Ca^{2+} dye indicator requiring an excitation wavelength of 480nm and has an emission of 514nm with a Kd for Ca^{2+} of 320nm.

The Ca^{2+} oscillations resulting were measured against those triggered in a parallel set of mouse eggs injected with the same dose of Nus-A hPLCζ protein in the same day. In individual eggs the injection dose was identified to be sufficient when a cytoplasmic displacement of 3-5% was seen during the injection. However, due to size and volume differences between both species, dose control was essential to provide comparable information for the resulting Ca^{2+} spikes. To achieve this, a co-injection of a fluorescent dye known as Alexa dextran 568 along with the protein was done, and this allowed for measurement of mean fluorescence intensity of the injection solution. Alexa dextran 568 is known to be greatly resistant to photo-degradation and pH insensitive over a large range (4-10) (Panchuk-Voloshina et al., 1999). A total of 40 human eggs from 14 different patients were made available to the project by the end. A breakdown of egg types and the IVF procedures that have been done for used eggs are presented in table 4.1 and table 4.2.

Egg type	Age	Number
MII	Day 1 post IVF	28 (70 %)
MI	Day 1 post collection	3 (7.5%)
GV	Day 1 post collection	9 (22.5%)
Total	40 eggs	

Table 5.1 Categorisation of human eggs used in the project.

The above shown table represents the breakdown of the human eggs that were used for this part of the project. They ranged from MII eggs, MI and GV eggs. About 70% of the eggs were MII.

Egg type	IVF	ICSI	No procedure
MII	13	10	-
MI	-	-	3
GV	-	-	9

Table 5.2 IVF procedure type applied to human eggs prior to micro-injection.

This table represents the type of IVF procedure that the eggs used were exposed to all eggs that were used were Day 1 post procedure. MI and GV eggs had no procedures applied to them.

As seen from the tables above, most of the human eggs were MII. Out of the 28 available MII eggs, 5 were received damaged or else in a poor state and were discarded as per the approved protocol. In this set of experiments, eggs were injected with a pipette concentration of Nus-A hPLCζ (0.125mg/ml) mixed with the Alexa dextran dye (0.2mM) as a co-injection solution. All eggs were imaged for a period of 10 hours and kept on the imaging system on the heated stage to follow any developmental changes the next morning. The response of injecting Nus-A hPLCζ into human eggs was variable. There was no specific pattern regarding the Ca^{2+} oscillations that were triggered. Except for the first experiment on human eggs, all human eggs and mouse eggs were injected with the same batch and concentration of Nus-A hPLCζ protein (1.25mg/ml). The MII human eggs exhibited 3 main categories of response. The first, was an instant start of high frequency oscillations (4-74 Ca^{2+} spikes) in the first 4 hours and it showed in 5/28 MII eggs

(17.9% of MII eggs), the next was a single/few Ca^{2+} spikes that started immediately (1-5 Ca^{2+} spikes) in the first 4 hours. This response was detected in 7/28 of the MII eggs (25% of MII eggs). In the second group there was only one egg that had a delayed start after 3.07 hrs.

The last group of eggs showed an atypical response in which mostly it had a delayed start (8/28, 28.6% of the MII eggs). In this group, the Ca^{2+} baseline was increasing throughout the imaging period and did not come back later. The Ca^{2+} traces of the last group bear a resemblance to that from the MII egg that were not injected and were imaged as controls. Out of the 28 MII eggs 6 were not alive the next morning (21.4%). Unfortunately, only two eggs (both were post ICSI) managed to reach a 2-cell stage the next day, 9/28 (32.1%) formed pronuclei and the rest (11/28, 39.3%) had no signs of any developmental progression (Fig.4.1, 4.3, 4.4). All MI eggs (3/40) had an atypical response where there was an ever increasing level of the baseline Ca^{2+} level, but all 3 remained alive the next morning (Fig.4. 2).

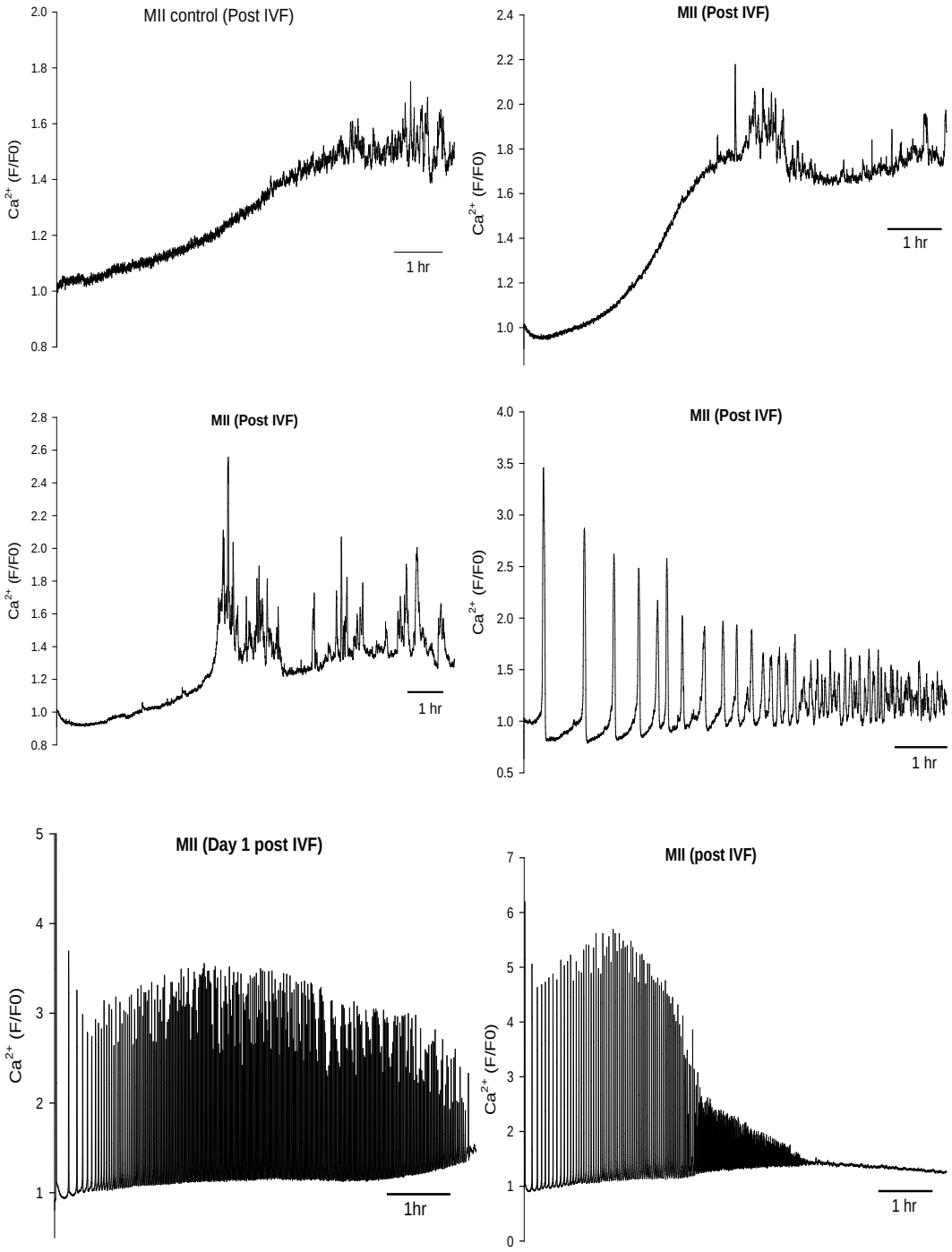

Figure 4.1 Ca^{2+} traces representing oscillatory pattern detected following a microinjection of hPLCζ into MII human eggs.

Nus-A hPLCζ was micro-injected in a concentration of 0.125mg/ml in all eggs.

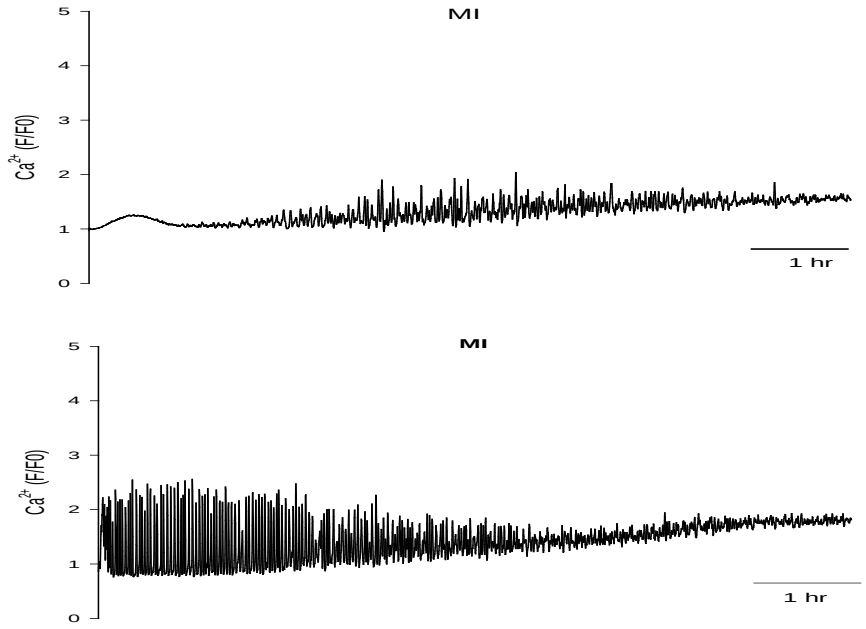

Figure 4.2 Traces of the Ca^{2+} indicator detected in human MI eggs post hPLCζ microinjection. (Nus-A hPLCζ conc. 0.125mg/ml).

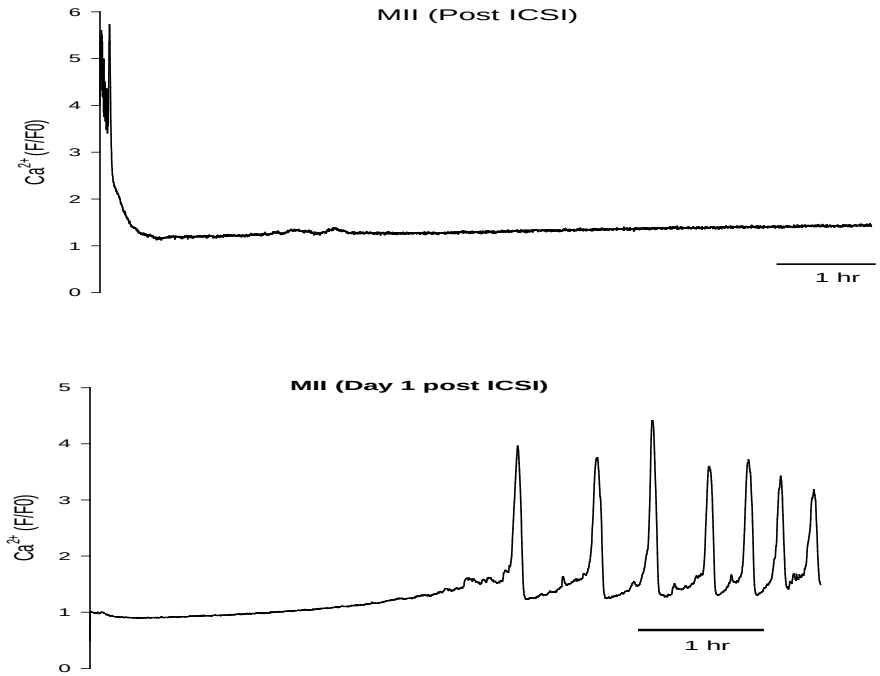

Figure 4.3 Effect of hPLCζ microinjection into MII human eggs.

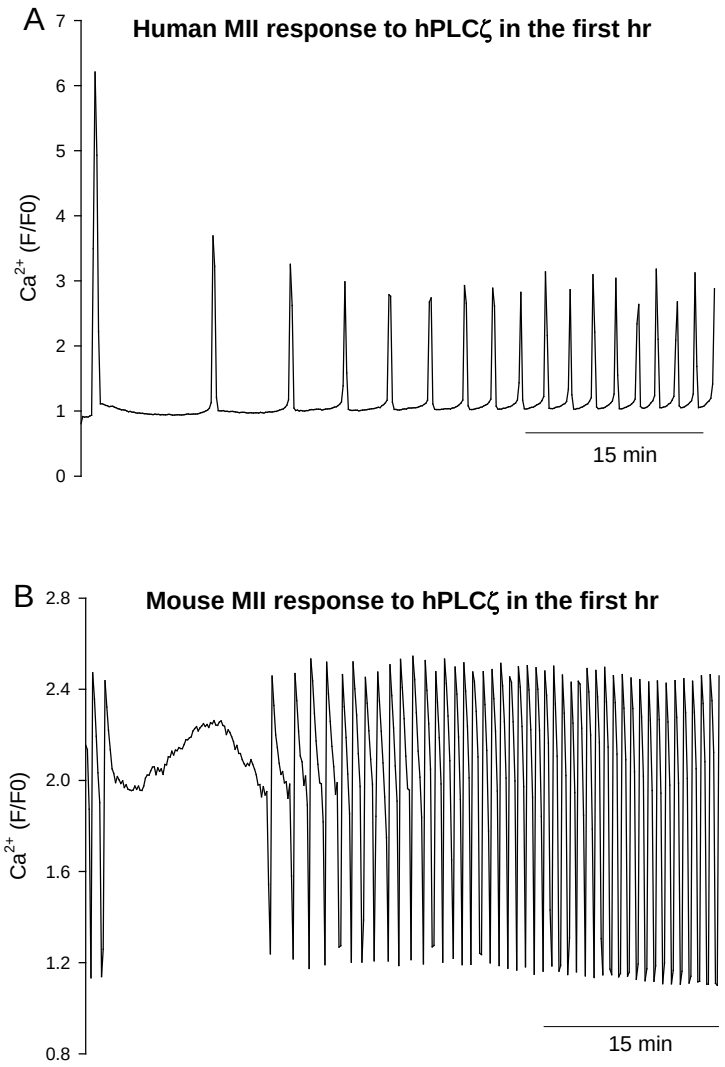

Figure 4.4 Ca^{2+} response after a micro-injection of Nus-A hPLCζ in M2 eggs (Human and mouse).

Human eggs **(A)** and mouse eggs **(B)** were micro-injected with the same dose of the protein (0.125mg/ml). The traces were cut after the first hour of imaging to reveal the difference visually. Bar scale=15 minutes.

4.3.2 Do human eggs arrested at GV stage have a specific response to a PLCζ protein injection?

The GV stage is one of the two physiological arrests that occur in mammalian oocytes (Mehlmann, 2005). A number of approaches have been attempted to release the human GV's from their arrest such as extending the period from hCG until retrieval, increasing the hCG dose and extending the period of in-vitro culture (Chian et al., 2000, Chian et al., 2004). In some mammalian models the size of the follicle during collection would indicate its ability to mature in-vitro (Motlik et al., 1986). However, in a human egg that is not always the case, as the follicle could be in the appropriate size range while the egg could be still developmentally retarded (Chen et al., 2010c). A number of studies reported on the effect of injecting either PLCζ cRNA or the sperm factor in mouse GV oocytes. The injection of cRNA led to a diminished response in triggering Ca^{2+} oscillations. The sperm factor injection was reported to be more successful in stimulating a response similar to that seen during fertilisation (Abbott et al., 1999, Wakai et al., 2013).

No studies on human GV oocytes exist in this regard. Therefore, with the remaining eggs which were at the GV state (9 oocytes) which were almost 25% of the total. It was thought of studying the effect of injecting them with Nus-A hPLCζ recombinant protein. It is acknowledged that the number of GV oocytes will not be sufficient to build any conclusions. However, the results will aid in planning any future projects. 5 oocytes that were at the GV state had an atypical response in which there were no Ca^{2+} oscillations while the baseline was drifting upwards during the period of imaging, the next day 4 GVs were not alive and one still had an intact GV. The next response that was noticed was when a GV started off oscillating in a manner seen during fertilisation with an initial 3 Ca^{2+} spikes. This pattern did not last, as afterwards it went into a heavy low amplitude oscillatory pattern. This pattern could probably be explained by the diminished internal Ca^{2+} stores present in this type of oocytes. The next day the GV remained alive with an intact germinal vesicle. The most interesting finding in this particular group was seen in one GV oocyte. Initially the oocyte responded with a first Ca^{2+} spike and then it remained in a quite phase for about 2 hours, after which it returned to oscillate in very similar pattern to that seen during fertilisation (Fig.4. 5). A total of 7 spikes were recorded during a period of 10 hours, the next day the GV had matured into an MII. Unfortunately, whether the oscillatory pattern was related to the GV breakdown or not, such a conclusion was not possible to make. DIC images were not taken alongside the fluorescence

imaging as it was thought that the extra exposure would lead to more stress on the oocytes.

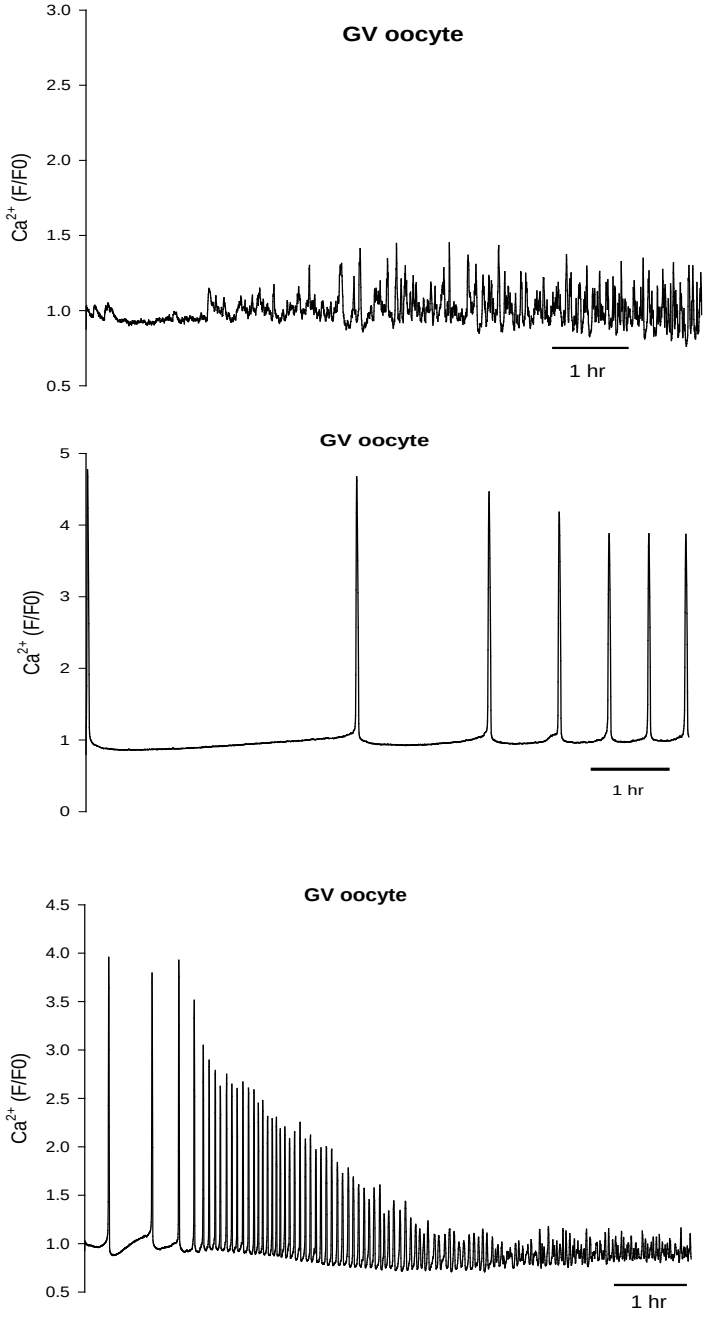

Figure 4.5 representative traces for Ca^{2+} changes detected in human GV oocytes following a microinjection of hPLCζ.

The GV oocyte with the middle trace was followed the next day and found to have matured into an MII. The dose of hPLCζ micro-injected was 0.125mg/ml in all GV oocytes.

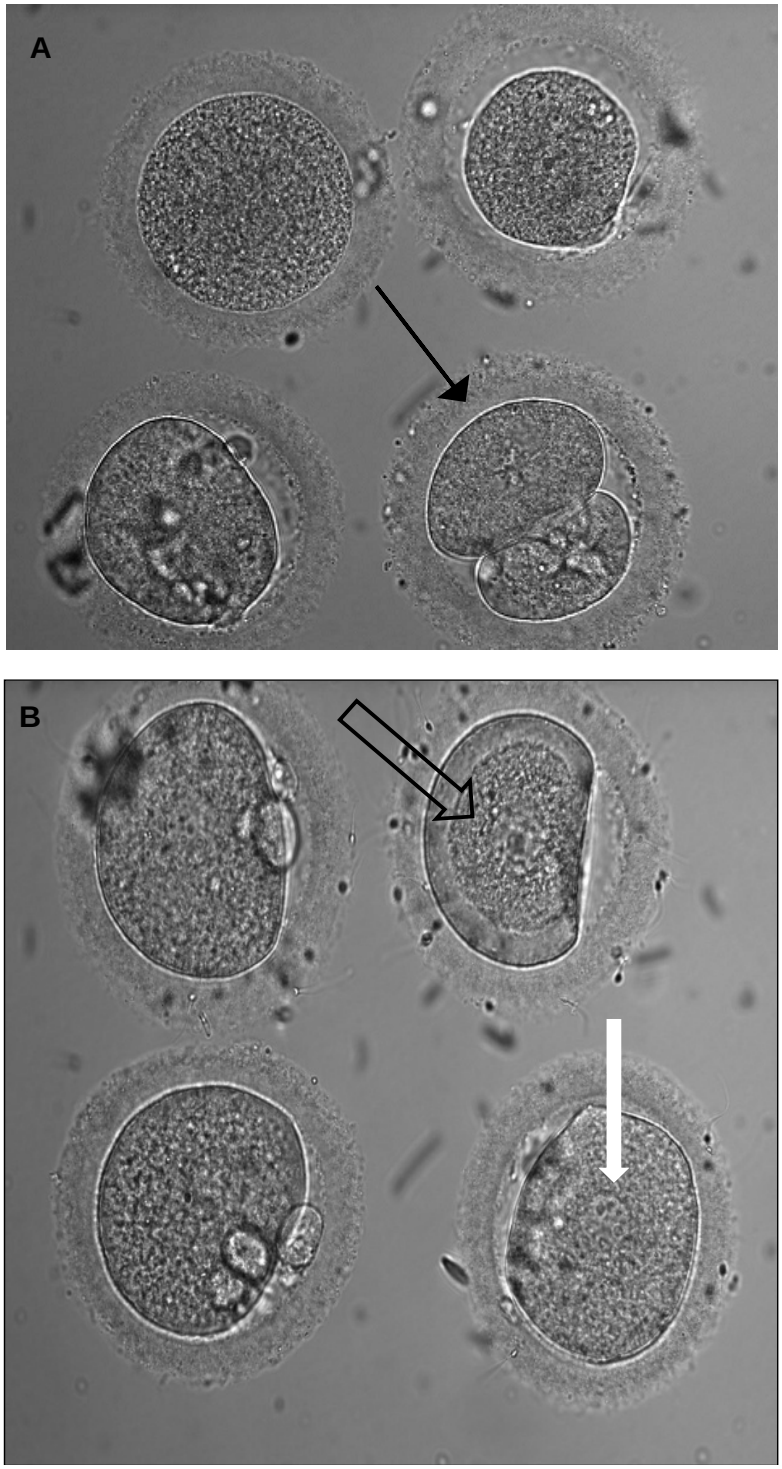

Figure 4.6 DIC images are for human eggs 24hrs post injection with hPLCζ protein.

A). The egg on the top left hand is showing signs of degeneration, while the egg with the black block arrow has gone through the first division into 2 daughter cells as a response to protein injection, the other two eggs have remained stationary.B).In this image the open black arrow shows a human GV oocyte that has been injected but remained post injection in the GV state, while the open arrow shows an MII egg that formed a pronucleus post injection.

4.3.3 A comparison of injecting a dose of hPLCζ protein into mouse eggs and human eggs that failed to fertilise.

A number of tests are available to assess the functionality of sperm after cases reported with fertilisation failure especially following an ICSI procedure. These tests include injecting sperm into other mammalian eggs such as mouse eggs (mouse oocyte activation test, MOAT). In this test the eggs are imaged to examine the pattern of Ca^{2+} oscillations as well as to check for pronuclei development in some instances. Three main categories were listed following such an examination, low, intermediate and high levels of activation capacity of the sperm (Heindryckx et al., 2005, Heindryckx et al., 2008). One main point was taken into consideration when analysing such results, that is the human PLCζ has greater potency than the mouse version (Nomikos et al., 2012a). In this set of experiments hPLCζ protein was injected into mouse eggs on the same day that the human eggs were injected. The exact same protocol used on human eggs was applied to mouse eggs. PLCζ protein was kept in -80^{o}C and only thawed prior to the experiment to prevent any decay in the activation capacity. A pipette concentration of PLCζ (0.125mg/ml) mixed with Alexa dye (0.2mM) was used to inject mouse eggs. Each experiment conducted on human eggs had a parallel experiment on mouse eggs using the same batch of hPLCζ (Fig.4. 7). The difference was that mouse eggs experiment was done on the morning using freshly ovulated mouse eggs. The human eggs were received usually at Day1 post IVF procedure at around 2pm (around 30hrs after collection). Each mouse egg experiment included between 6-9 eggs being injected and imaged. A total of 72 mouse eggs were injected and analysed. All mouse eggs responded to the Nus-A hPLCζ protein injection (Fig.4. 7 A). The injection process for 6-9 mouse eggs usually takes less than 1minute, the response was mainly characterised by a high frequency of Ca^{2+} oscillations. The mean spike interval of the oscillation was 4.3+_ 0.48 (s.e.m) mins (Table 4.3) and there was no gap or delay between placing the eggs on the imaging system and the start of the Ca^{2+} oscillations. In most mouse eggs the first Ca^{2+} spike was missed due to the rapid onset of action of the protein. In about 28/72 (38.9%) of the mouse eggs the high frequency of Ca^{2+} oscillations showed an abrupt cessation after about 2.5hours without any reduction in the amplitude or frequency beforehand. Along with the Nus-A hPLCζ, 5μMAlexa indicator was co-injected to allow for measurement of fluorescence intensity in eggs that have been micro-injected. The fluorescence intensity of the Alexa dye was used to compare the amount of the protein injected in human and mouse eggs. Figure

4.7(C) demonstrates that the amount injected into mouse eggs (12.9-31.5) was about half of what was injected into human eggs (39.7-66.2). add to disc why

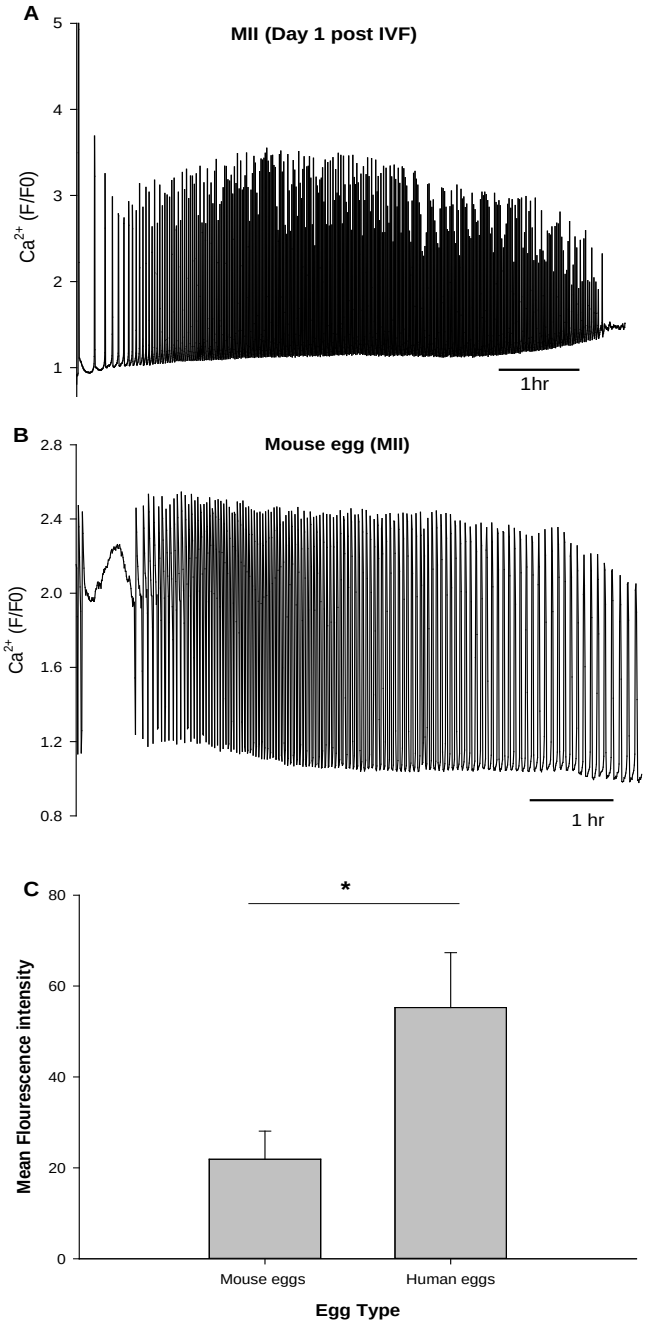

Figure 4.7 A comparison of Ca^{2+} oscillations between mouse and human eggs following a micro-injection of hPLCζ.

Both egg types received an injection of hPLCζ of 0.0125mg/ml. (A, human egg. B, mouse egg). C, The bar chart reveals a significant difference between the amount microinjected into both eggs (*P*= 0.000).

4.3.4 PLCζ protein activation capacity can be preserved by freeze drying.

Until now the evidence compiled from the work on PLCζ is strongly indicating that it can be used as an alternative for assisted activation protocols in IVF clinics in the near future. However, the protein has been studied in its solution form in terms of duration of activity when taken out of -80^{o}C. If the protein is to be used as a therapeutic agent it will need to be distributed and in its current form it will need a reliable cold storage which in part could be expensive and not always available especially in developing countries. Freeze-drying has been used for decades as a process for concentrating or drying biologically active enzyme/protein based materials. Freeze-drying also offers a big advantage when it comes to shipping/distribution and storage (Roy and Gupta, 2004). However, recently it was noted that the reduced activity of some enzyme powders was related to the freeze-drying process. It is of great importance to ensure the stability of PLCζ protein during this process of form modification, transport and storage. In view of these concerns it was mandatory to test the effect of freeze-drying on PLCζ protein and its potential for recovery after reconstitution.

For this preliminary test of freeze-drying of Nus-A hPLCζ protein 2 new stocks were made for this purpose. The first was to test for Ca^{2+} releasing activity post freeze-drying and the second was to test if storage effects on egg activation capability of the protein. First was a batch that had a concentration of 0.9mg/ml, which was divided into aliquots of 5µl each. It was tested on the same day of purification at a concentration of about 0.01mg/ml (group 1). The rest of the aliquots were subject to freeze-drying on the same day and were kept at 4^{o}C for less than 24hrs (16hrs +/- 4hrs). The next day the protein was reconstituted and divided into two aliquots, one aliquot was used to inject eggs after a period of 1hour while the other was kept at 4^{o}C (group 2). Next, mouse eggs were injected with hPLCζ protein that remained at 4^{o}C after reconstitution, the protein was diluted to the required concentration (0.01mg/ml) before injection (group 3). Table 4.3 summarises the result of the pattern of Ca^{2+} oscillations for the three groups injected. When comparing the response elicited from injecting PLCζ in the same concentration of the 3 different conditions, it seems that the higher activation capacity was that of the third group. Traces in figure 4.7 are representative of the 3 groups. The mean number of Ca^{2+} spikes was significantly different between the 2 groups that were freeze dried, with the second group having the highest rate. In group 2 there were about 33% (4/12)

of mouse eggs that did not show any response to PLCζ while the rest of the eggs started oscillating after a gap of at least 1 hour. Eggs in group 1, 3 started oscillating immediately with no gap. This data might suggest that freeze-drying might have promoted aggregation of the protein that requires time to fully reconfigure after reconstitution. However, it has to be noted that we found that once the protein is diluted to the required working concentration it loses its ability to induce any Ca^{2+} oscillations after 24hrs.

State of PLCζ	**Response in mouse eggs Mean spike number /4hours +/-Std**
(a) PLCζ injected on Day 0 (on purification day)	6.14 (n=14) **+/-**6.2
(b) PLCζ injected on Day 1 post freeze-drying (1 hr post reconstitution)	1.83 (n=12) +/- 4.8
(c) PLCζ injected on Day 2 post freeze-drying (day 1 post reconstitution)	8 (n=10) +/- 2.9

Table 5.3 Effect of freeze drying hPLCζ protein and its ability to trigger Ca^{2+} oscillations following different thawing conditions.

The above table provides details of micro-injecting hPLCζ **(a)** on the day of purification and **(b)** post freeze drying and reconstitution and dilution at Day 1. **(c)** Injecting mouse eggs on Day 2 post reconstitution after dilution. A significant difference was detected between groups that were freeze dried (P= 0.007).

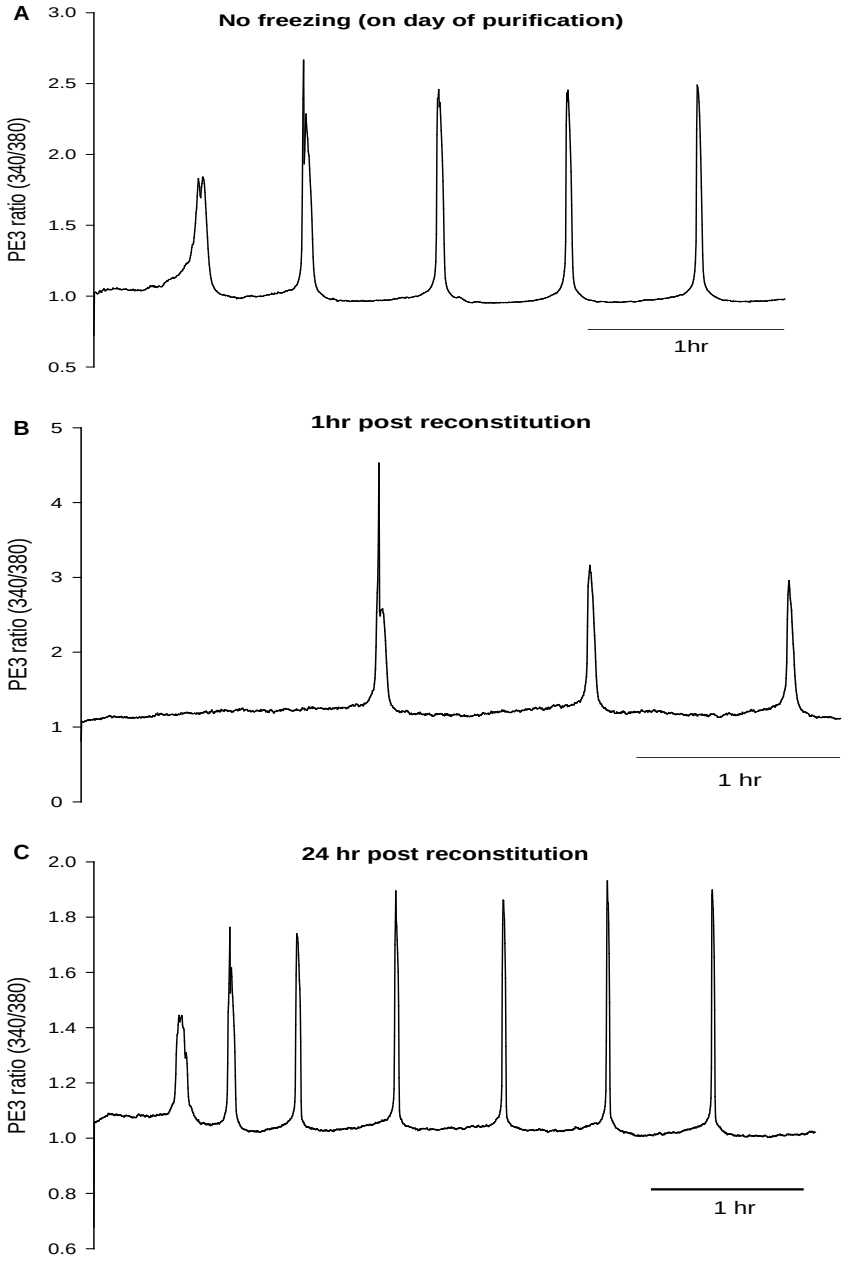

Figure 4.8 Ca^{2+} oscillations established in mouse eggs following injection of hPLCζ subject to different storage methods.

A, A Ca^{2+} trace resulting from injection of the protein into a mouse egg on the same day of purification. **B**, Following freeze drying the protein was reconstituted to its original concentration then diluted to the working concentration required. **C**, Following reconstitution to the original stock concentration the protein was left at 4^{o}C and diluted to the working concentration and micro-injected into mouse eggs the following day. All eggs were micro-injected with 0.01mg/ml of hPLCζ protein.

Next to assessing the stability of the protein after being freeze-dried and reconstituted, it was necessary to ensure that storage would not compromise the activation capacity of the protein. It has been reported that freeze-dried proteins are liable to denaturation in certain conditions by moisture and other mechanisms (Roy and Gupta, 2004). For this purpose the second batch was used to identify if protein storage would have any effect on its activity. The second purified protein batch was 30µl with an initial concentration of 1.25mg/ml. The stock was divided in three groups the first 10µl was divided into 2 of 5 µl aliquots and stored at -80^{o}C after snap freezing using liquid nitrogen. The second group was divided into 2 of 5 µl and freeze-dried on the same day, post freeze drying the protein was kept in laboratory condition with a temperature ranging between 21^{o}C - 23^{o}C inside a drawer. The third group was also divided into 2 of 5 µl aliquots and freeze-dried on the same day and kept at about 4^{o}C. All groups were stored for 4 weeks after which the protein was tested by injecting different concentrations (0.15mg/ml - 0.08mg/ml - 0.02mg/ml) into mouse eggs to identify if any differences would exist in the response elicited.

There was no significant difference in the mean spike number per 2 hours detected between the 3 groups when a concentration of 0.15mg/ml was injected into mouse eggs (Table 4.4) (Fig.4. 10). When 0.08mg/ml was injected into eggs the highest oscillation frequency detected was seen in the group that was stored at 4^{o}C and that was significantly different from the other 2 groups (P= 0.000) as illustrated in figure 4.9 and figure 4.11. When the concentration of injection was further reduced to 0.02mg/ml the only group that showed a response to the injection was the group that was injected with the protein that was stored at -80^{o}C (Fig.4.12). The other two groups representing the frozen-dried protein did not trigger any response in mouse eggs injected.

The results show that at high concentrations of the protein no difference exists between different modes of storage. However, when the concentration was lowered it seemed that the form of protein storage could possibly affect the activation capacity in that freeze-drying stored at 4^{o}C was significantly superior in retaining the protein activity. With the lowest concentration of protein injected (0.02mg/ml) the form of storage that elicited a response was the one kept at -80^{o}C indicating that long term storage could affect the protein activity.

Protein concentration injected	Mean spike number/2hrs		
	FD kept at room temp	FD kept at 4°C	Kept at -80°C
0.15mg.ml	89 (n=8)	78.5 (n=8)	74 (n=8)
0.08mg.ml	6.5 (n=6)	46.4 (n=8)	8.5 (n=8)
0.02mg/ml	0 (n=6)	0.13 (n=8)	3.2 (n=9)

Table 5.4 Ca^{2+} spike number following injection of various concentration of hPLCz that have been stored via different methods.

The presented table shows the mean number of Ca^{2+} spikes imaged for the first 2 hours for the 3 different concentrations injected of the 3 different methods of protein storage. (FD, Freeze dried).

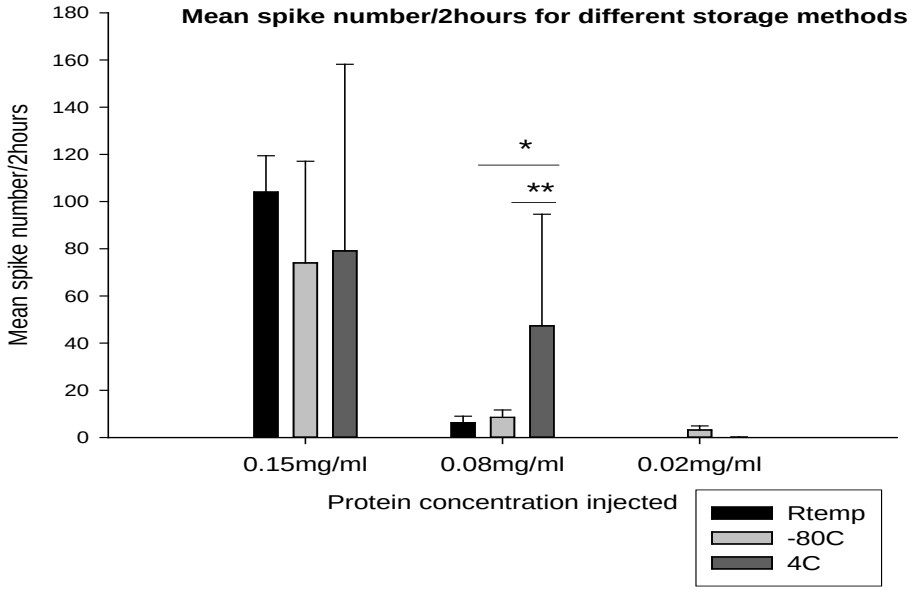

Figure 4.9 Bar chart representing the mean number of Ca^{2+} spikes difference between various storage methods for the 3 different concentration of hPLCζ micro-injected.

There was a significant difference detected for the means of spike numbers in the 0.08mg/ml concentration for the 3 different storage methods (*P*= 0.000)

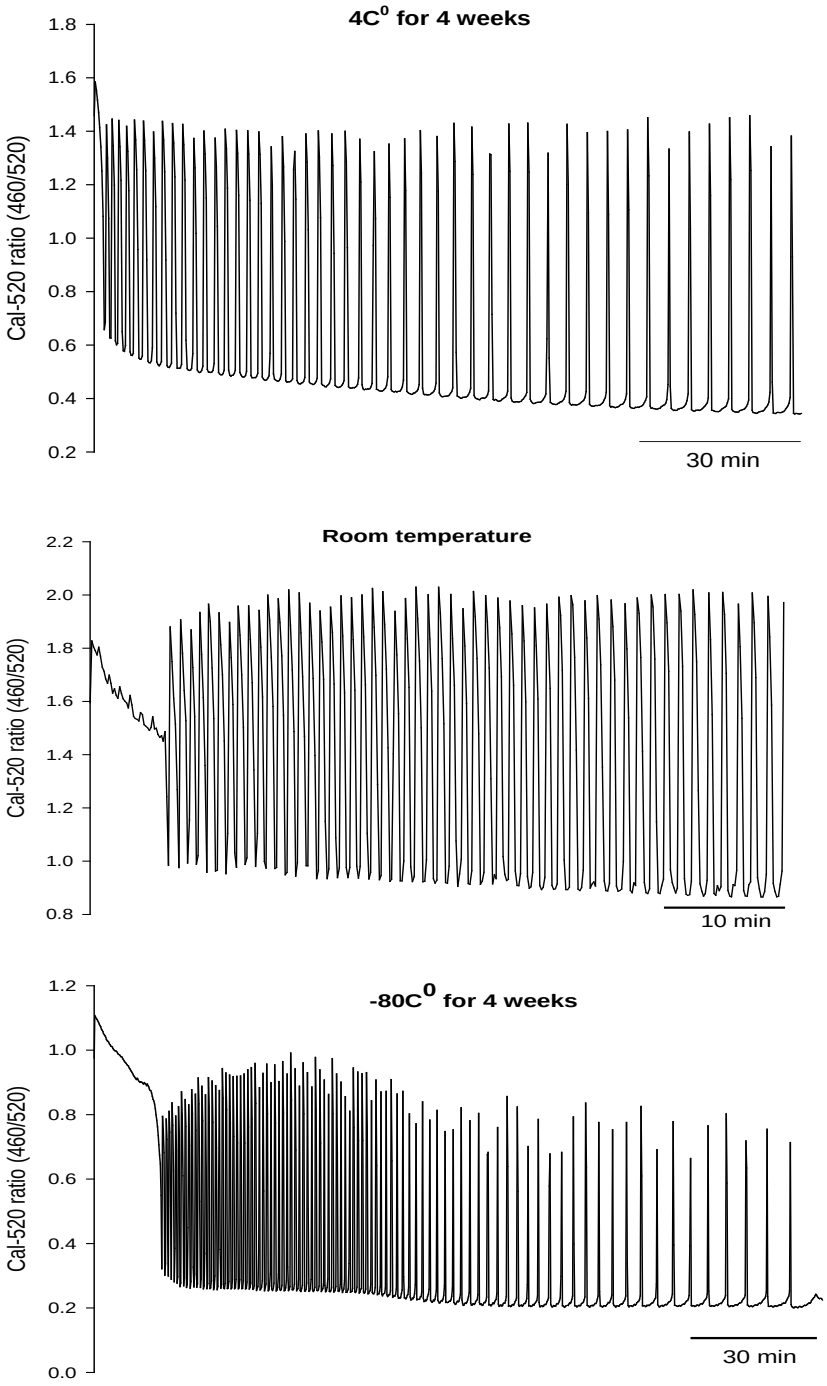

Figure 4.10 Sample traces for Ca^{2+} oscillations in mouse eggs in a response to a microinjection of hPLCζ (0.15mg/ml).

A hPLCζ concentration of 0.15mg/ml was microinjected into mouse eggs from the 3 different storage methods used for the protein.

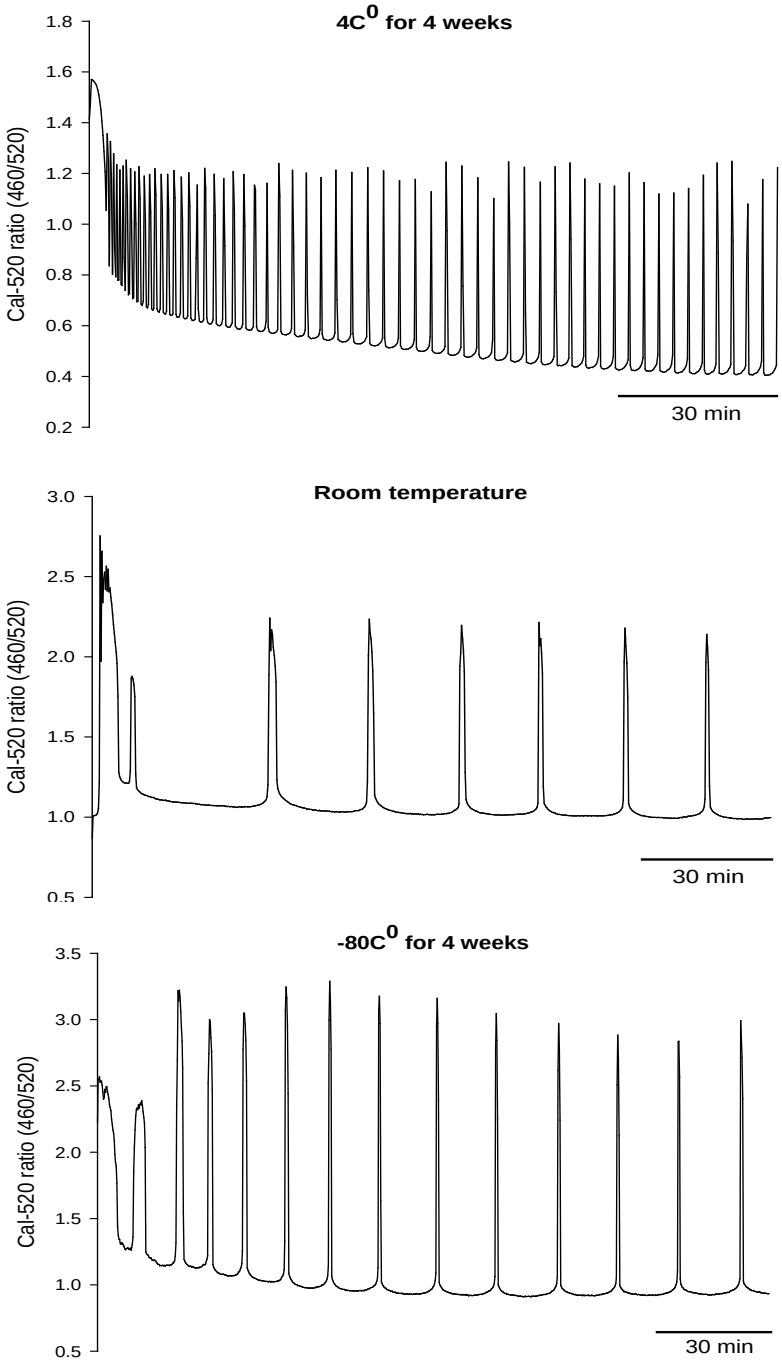

Figure 4.11 Representative traces for the Ca^{2+} oscillatory pattern detected in mouse eggs following a hPLCζ dose of 0.08mg/ml.

The three traces are representative Ca^{2+} oscillation traces detected for the different storage methods of the protein for the given concentration.

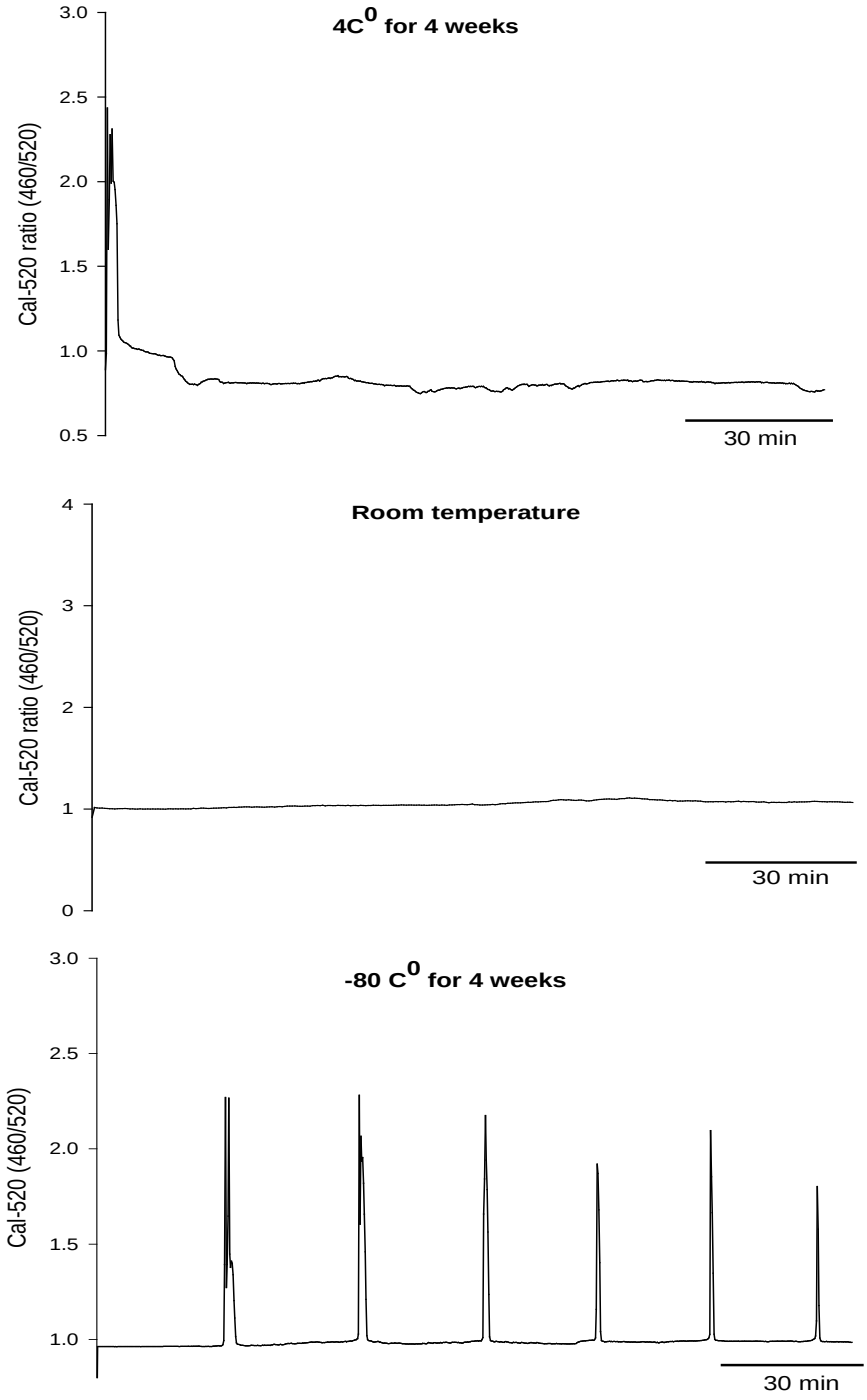

Figure 4.12 0.02mg/ml of hPLCζ microinjected into mouse eggs following various methods of storage.

Protein stored at -80^{o}C was the only reliable source of Ca^{2+} oscillations at this particular concentration and it was significantly different from the other two methods of storage (P= 0.001). Difference detected was between the mean spike number/2hrs.

4.3.5 Exploring methods for injecting PLCζ protein in IVF settings.

A number of approaches have been developed to micro-manipulate gametes during various IVF techniques. The latest most commonly used technique is ICSI which involves introducing a single sperm into the egg cytoplasm via the use of a sharp injection pipette. The downside of this technique is that it involves puncturing the cytoplasmic membrane which has the risk of irreversibly damaging the egg by lyses or shrinkage (Palermo et al., 2009). However, the learning curve of most embryologists led to optimal injections that had high survival rates. Loss of eggs when induced ovulation cycles yield a high number of eggs wouldn't be an issue, however, in cycles where the egg harvest is low in poor responders a suboptimal injection technique could be of clinical relevance (Ebner et al., 2001). Current artificial activators such as ionomycin are used in way that does not involve mechanical intervention with the eggs, therefore reducing the risk of immediate damage to eggs. In light of the potential use of Nus-A hPLCζ protein as an artificial activator in clinical settings, it is important to find a method that would allow its introduction into egg cytoplasm without the risk of egg damage. To use PLCζ protein as an activator it be would require a suitable injection technique to be established. In this section we explored a number of techniques to identify a reliable method that would have the lowest risk in irreversibly damaging the eggs.

4.3.6 The effect of pipette type on hPLCζ protein injection.

In our laboratory settings we utilise a high pressure (>10 psi) injection system for injecting hPLCζ protein which uses an electrically driven sharp tip (~1µm) pipette after and ICSI injection in mouse eggs. In order for us to achieve this, two types of injection pipettes are needed, an ICSI pipette and the sharp tip pipettes. Both pipettes are different from that used in IVF clinics

It was initially thought that hPLCζ protein could be injected as a second step in a process involving two subsequent measures. For that purpose a specific pipette was fabricated using a pipette puller, and a beveller to obtain the desired tip geometry. The idea here is that a second pipette could be used that mimics our procedure but which is wide enough to be used with low pressure injection apparatus typical of IVF clinics. The bevelled tip was expected to facilitate the penetration through the zona pellucida while the tip diameter would be sufficient to release protein once inside the egg cytoplasm. The protein was placed in a separate drop to that containing the eggs placed adjacent to one another so that movement between drops would not be

problematic. There was no contact between both drops as they were bridged by the covering mineral oil. To examine the ability to carry a specific amount of protein and to inject it into eggs without diffusion from the end of the pipette, a fluorescent-dextran was used as a tracer for this technique. The injection was carried out without the use of the high pressure as the system was unfamiliar to IVF clinics. The bevelled pipette was connected to the ICSI system and the protein was injected manually. By measuring the mean level of fluorescence of the indicator post injection we can see that using such a technique could be reliable in injecting comparable amounts of material across the eggs. The injection using a bevelled pipette was compared to that of utilising a standard high pressure mouse injection pipette (Fig.4.13)

No statistical difference was identified between eggs in each group (*P*= 0.275) and when the mean fluorescence level was measured between both groups no difference was statistical significant difference was detected either (*P*= 0.096). However, this was technically quite demanding as the control of the uptake of the fluorescent material was difficult to keep in the bevelled pipette once carried over to the injection media. It seemed as there was a constant diffusion from the pipette tip into the injection media drop. Initially the drop that had the fluorescent dextran was mixed with KCL Hepes. When the drop was supplied with 10% polyvinylprolidine (PVP), it was hardly possible to take it up into the injection bevelled pipette. PVP is commonly utilised in the sperm suspension drop to reduce sperm movement in IVF clinics by increasing the viscosity of the drop (Van Steirteghem et al., 1993). From this experience, I can conclude that the control of the amount of material injected under these experimental conditions into egg cytoplasm could be equally achieved via the use of both pipettes but with a notable degree of technical difficulty.

The use of a single ICSI pipette would be favoured over the use of the second bevelled pipette, but this means it will be necessary to develop a co-injection method for sperm and protein. The concept of a co-injection would be ideal, as it would avoid exposing the eggs to a second round of mechanical manipulation that would be required by the use of the extra bevelled pipette. The ICSI technique basically involves including a sperm in the injection solution, therefore including sperm in a suspension was tested next. The setting remained the same as in the previous ICSI work, but with the addition of sonicated sperm to the drop containing fluorescein dextran as a fluorescent indicator. The comparison of amounts injected

was made between using a pipette with our usual high pressure injection method and the mouse ICSI pipette method. Once again, the experiment revealed that sperm could be co-injected into eggs with an amount of the indicator that showed no significant difference across the mean of fluorescence levels detected in both groups of eggs injected (P= 0.083) (Fig.4. 14).

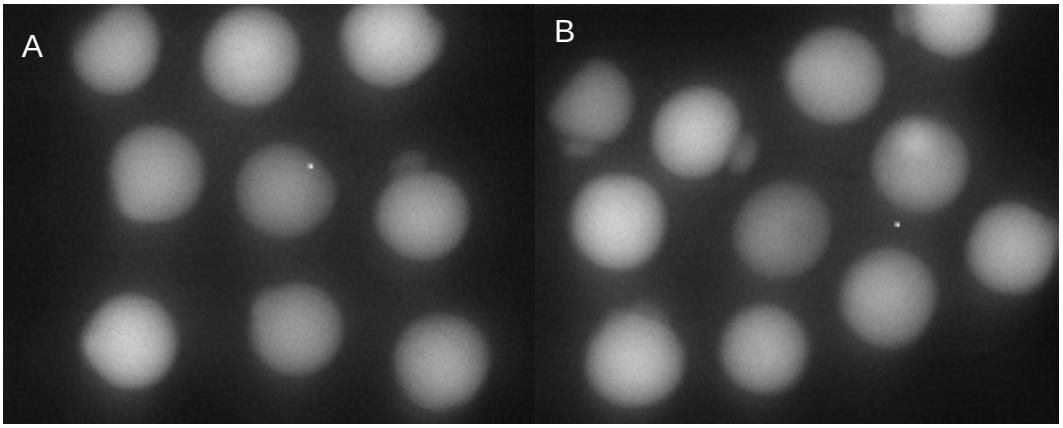

Figure 4.13 Fluorescence images for mouse eggs following injection of fluorescein dextran using different pipettes.

A, Eggs microinjected using a pipette for the high pressure injection system. **B,** Mouse eggs injected using a bevelled pipette.

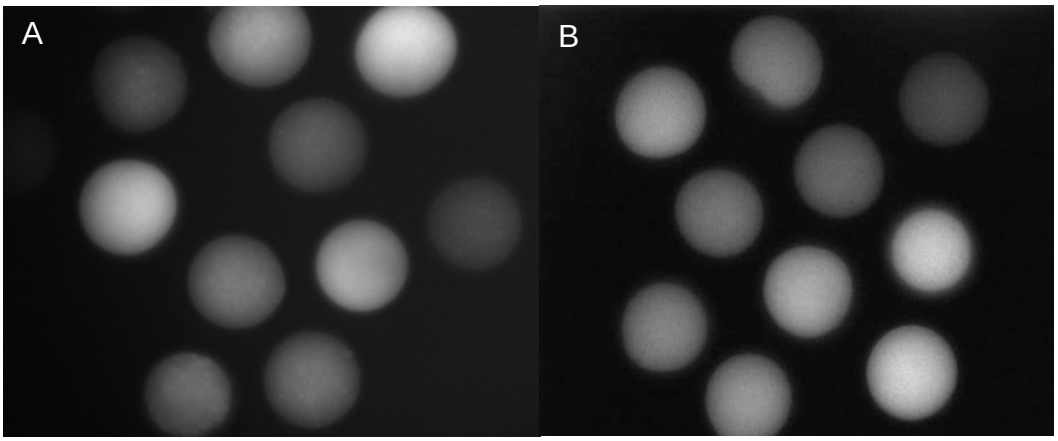

Figure 4.14 Fluorescence images of mouse eggs post injection with fluorescein dextran.

A, Microinjection using a pipette that is used with high pressure injection. **B,** Mouse eggs injected using a mouse ICSI pipette.

4.3.7 Exploring media that would be suitable for a co-injection method.

The ICSI protocol involves a setup where one drop of media will contain the eggs and other drops containing the sperm that will be manipulated prior to pick-up into the pipette. Following the holding of a sperm, the ICSI pipette will be moved towards the drop where eggs are placed and the injection of sperm will there take place. This coming set of experiments was to find the best place to add PLCζ protein and which media would provide the best survival rates post injection. The initial consideration was to have PLCζ protein at the concentration required (0.015mg/ml) within the media that contains the eggs. As most of our injection procedures are conducted in M2 media, it was the first media to test. Having PLCζ protein in the injection media proved to have deleterious effects on egg survival when a sham ICSI procedure was done (with no sperm). Only 4 out of 18 eggs survived the procedure and those that survived had a very high frequency of oscillations when imaged. Next, eggs were kept on their own in M2 medium drop. PLCζ protein was diluted to the required concentration in a separate KCL-Hepes drop. Both drops were next to each other separated via a bridge created by the covering mineral oil. The same results occurred, in that only 3 out of 12 eggs survived the injection and those that did survive had very high frequency oscillations. These data suggest that simply combining PLCζ protein with the normal media surrounding the egg is not an effective means of introducing it into eggs.

The Ca^{2+} in M2 was suspected to be the main culprit behind the over-stimulated response of eggs and their lysis. PLCζ is notably very sensitive to Ca^{2+} and could be hyper-stimulated by the Ca^{2+} in media. . Because M2 is a ready bought media from Sigma we switched to KCL-Hepes, which was necessary to be able to manipulate the contents of the injection media and to test the effects of certain molecules. To this media 1mg/ml of PVP was supplemented to prevent the sticking of the eggs to the bottom of the injection dish. Eggs and PLCζ protein were then placed in the drop at a concentration of (0.015mg/ml) and ICSI was performed. Ca^{2+} was omitted from the media at first but that caused lyses of all eggs after injection (survival 0/16). Next Ca^{2+} was added to reach a concentration of 10µM and there was a significant improvement in survival from the previous result (P= 0.002) with 15 out of 27 eggs surviving the injection. However, the frequency of oscillations was still high (7.5 times the physiological rate). The concentration of Ca^{2+} was further increased to 100µM. The survival rate was improved when compared to the first attempt, but significantly less than the group injected with 10µM of Ca^{2+} media (P= 0.0032), with

just 6 out of 21 eggs surviving the injection. These data suggest that extracellular Ca^{2+} is needed for survival after ICSI, but even a small amount of extracellular Ca^{2+} causes problems when co-injected with PLCζ protein.

HKSOM is a culture media that has components suitable for development and is made in our laboratory so adjustments can be applied. As a control for this group regular HKSOM media with 1.7mM Ca^{2+} was used for a regular ICSI experiment where sperm was placed in the drop with eggs. The survival rate was 22 out of 26 injected eggs (84.6%) with 19 eggs (86.3%) having a physiological pattern of Ca^{2+} oscillation when imaged. Subsequently, HKSOM that had no Ca^{2+} addition was used for an ICSI run, but all 14 eggs of the first attempt stuck to the bottom of the dish. For that reason it was necessary to add bovine serum protein (BSA) which is a common blocking agent to prevent adhesions. So 4mg/ml of BSA was added to HKSOM, with no Ca^{2+} and a sham ICSI was performed where no sperm or PLCζ protein. The run was repeated 3 times where a total of 37 eggs were injected but 0% of eggs survived the injection. Ca^{2+} was reintroduced to the media gradually starting from 10µM, and a sham ICSI was performed to test the survival and 18 out of 22 (81.8%) survived and looked healthy post injection. Sperm was added to the injection media and 25 eggs from a total of 28 eggs injected survived the procedure (89.3%). Sperm and PLCζ protein were added to the media with a final concentration of (0.015mg/ml) but no egg survival occurred at all after 3 separate attempts ICSI (Table 4.5, 6, 7 a,b,c). These data using HKSOM resemble those using KCl Hepes and again suggests that Ca^{2+} is important for egg survival and that eggs are stressed by the combination of Ca^{2+} and PLCζ protein injection.

A ICSI conditions		Survival rate	Oscillation pattern
Media	**Additions**		
M2	Control set ICSI with sperm	11/13 84.6%	Physiological
M2	Sham ICSI & PLCζ 0.015mg/ml	4/18 22.2%	Very high frequency
PLCζ protein in a separate drop (KCL-Hepes)	ICSI with sperm & PLCζ 0.015mg/ml	3/12 25%	High frequency

Table 5.5 Assessment of M2 as a medium for a protocol of co-injecting sperm and hPLCζ protein.

A, Variations in the co-injection protocol using M2 medium, including survival rates and pattern of Ca^{2+} oscillations detected.

B ICSI conditions		Survival rate	Oscillation pattern
Media	**Additions**		
KCL-Hepes	No Ca^{2+} 1mg/ml PVP 0.015mg/ml PLCζ	0/16	----------
	10µM Ca^{2+} 1mg/ml PVP 0.015mg/ml PLCζ	15/27 55.6%	High frequency
	100µM Ca^{2+} 1mg/ml PVP 0.015mg/ml PLCζ	6/21 28.6%	Very high frequency

Table 5.6 Search for a media that would allow maximum survival rates for mouse eggs post ICSI-hPLCζ co-injection.

B, Mouse eggs were placed in a drop of KCL-Hepes with various additions the table shows results of egg survival and Ca^{2+} oscillation patterns detected post injection.

C ICSI conditions		Survival rate	Oscillation pattern
Media	**Additions**		
HKSOM	1.7mM Ca^{2+} ICSI with sperm	22/26 (84.6%)	19/22 responded with physiological frequency
	No Ca^{2+} ICSI with 0.015mg/ml PLCζ	All eggs stuck to bottom of the dish	----------
	No Ca^{2+} 4mg/ml BSA Sham ICSI	No egg survival	----------
	10µM Ca^{2+} 4mg/ml BSA Sham ICSI	18/22 81.8%	----------
	10µM Ca^{2+} 4mg/ml BSA ICSI with sperm	25/28 89.3%	Physiological frequency
	10µM Ca^{2+} 4mg/ml BSA ICSI with 0.015mg/ml PLCζ	No egg survival	----------

Table 5.7 HKSOM as a medium for a co-injection protocol for ICSI and hPLCζ protein.

C, The table represents results from experimenting with different changes to the regular HKSOM, no physiological Ca^{2+} oscillations were detected apart from the control listed as the first test in the table and when no hPLCζ was included in the media.

4.3.8 The relationship between egg survival during ICSI-PLCζ co-injection and medium osmolarity.

Osmolarity can affect the size of the vitelline space by either shrinkage or swelling in a reaction to the surrounding medium osmolarity (Kaufman and Surani, 1974). Frog eggs are often injected in Ca^{2+} free media (to prevent egg activation) and it has been reported that survival of frog eggs after injection is improved by increasing the osmolarity of the media (Edwards et al., 2004). The normal range of osmolarity that experimental or culture media are expected to be is around 280-300mOsm/Kg, which simulates mammalian serum osmolarity (Waymouth, 1970). To investigate the effect of osmolarity, more specifically a high osmolarity on the survival rate of eggs during an ICSI- PLCζ co-injection. I deliberately made the osmolarity of the injection media so it will be higher than normal. The change to an increased level of osmolarity was induced by subtracting NaCl from the injection media (HKSOM) and replacing it with choline to achieve the desired osmolarity. Therefore all sodium salts were replaced by choline, measurements of the end-point osmolarity was checked using a calibrated freezing point osmometer. The new osmolarity ranged between 350-400mOsm/kg. Choline unlike sodium does not cross the cytoplasmic membrane by the use of channels and it has a membrane protection capability as shown by previous work on eggs (Stachecki et al., 1998).

When eggs were placed in the injection medium, a morphological change appeared in the size of the egg probably due to a change in the peri-vitelline volume. The result of placing the eggs into a higher osmolarity medium was felt in addition to being visualised. It seemed that the cytoplasmic membrane was toughened and was more resistant to needle advancement. However, despite the effect of increasing the osmolarity on the eggs particularly on the membrane, there were no beneficial effects detected in terms of egg survival. Part of the experiments conducted in HKSOM regular media were repeated with the high osmolarity version. The last two ICSI conditions in table 4.5 C were repeated and mixed results were found. The conditions were that where 10μM Ca^{2+}, 4mg/ml of BSA and either sperm or 0.015mg/ml of hPLCζ was present. In terms of survival there was a significant improvement when the experiments were repeated in different medium osmolarity. Egg survival of 14/20 (70%) (P= 0.016) for co-injection in HKSOM, 10μM Ca^{2+}, 4mg/ml of BSA and sperm was detected. Moreover, egg survival of 11/19 (57.9%) (P= 0.021) for co-injection in HKSOM, 10μM Ca^{2+}, 4mg/ml of BSA and 0.015mg/ml of hPLCζ was detected. This was significantly better in comparison with regular

media osmolarity. However, the improvement seen in survival rate was not seen in the normalization of Ca^{2+} oscillation frequencies. The rate of Ca^{2+} oscillations detected for the ICSI with sperm had a reduction of about 67% of the spike number/2hrs. While the group which had an ICSI- PLCζ protein co-injection only 3/28 eggs oscillated and that was in an unusual pattern as seen in figure 4. 15.

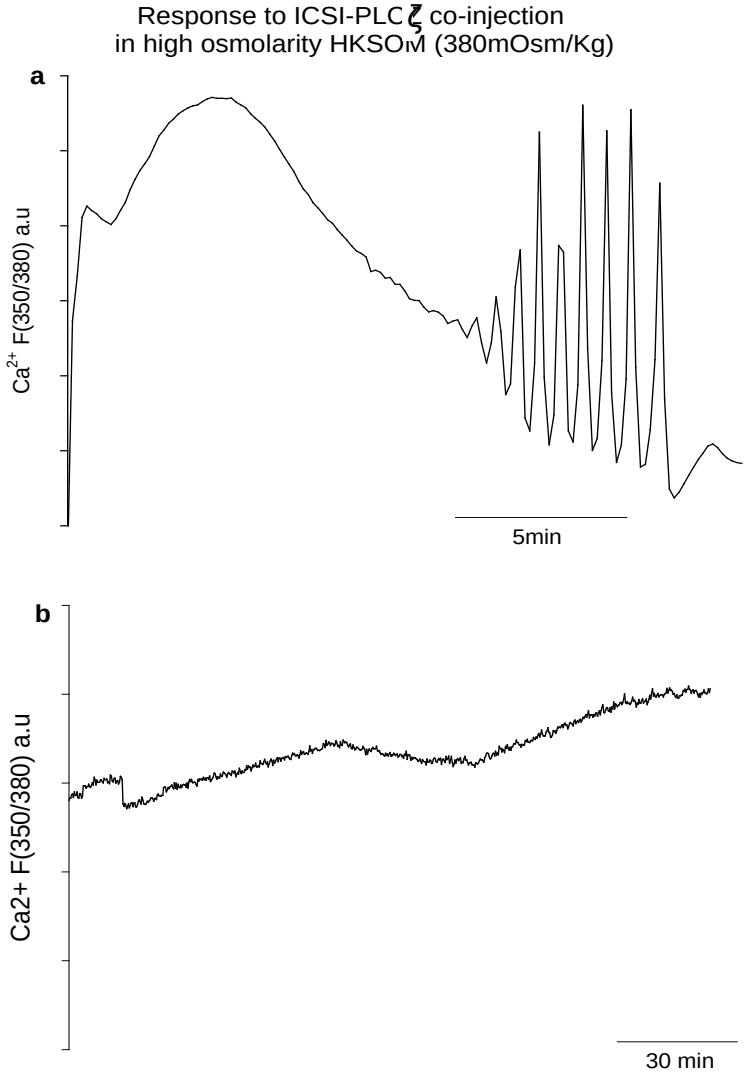

Figure 4.15 Representative traces for cytosolic Ca^{2+} changes following injecting hPLCζ protein in media with various osmolarity levels.

A, Represents an abnormal Ca^{2+} oscillatory pattern detected following hPLCζ injection. **B**, No Ca^{2+} oscillations detected in mouse eggs after injection.

4.3.9 Could a conventional method of ICSI injection be the solution?

Since development of the ICSI technique, it has not seen a dramatic alteration in the way it is has been performed. The basic protocol has almost remained unchanged throughout the years (Palermo et al., 2009). The technique, as described in details elsewhere, includes a dish with multiple media drops usually each containing a single egg to be injected. A separate drop for the sperm preparation is either located on the side or at the centre of the dish. Sperm preparation is usually supplemented with PVP, although controversy surrounds its use, it is still a common practice to reduce sperm mobility. In my previous experiments exploring media suitable for a co-injection technique, there was a diffusion issue of the material from the end of the injection pipette. This was noticed first when M2 medium was used with an adjacent KCL-Hepes drop containing hPLCζ. However, when 10% PVP was added to KCL-Hepes this phenomenon was reduced. For that reason, it was thought that going back to the original configuration of the technique could provide a solution to the diffusion issue. Therefore the initial setup was to test if the diffusion would be reduced if a PVP concentration used in IVF settings (5-10%PVP) was used in the separate drop.

A dish with a M2 drop containing eggs and another being KCL-Hepes supplemented with 10%PVP and a fluorescent heavy molecule (Fluorescein isothiocyanate–dextran ,FITC-dextran, sigma) were covered by mineral oil and placed on the injection stage. The vibrant yellow colour of the indicator allowed an instant assessment of any diffusion occurring after loading the injection pipette and moving it across to the injection media. It seemed that the viscosity difference between the two media brought by the use of PVP prevented the diffusion. Eggs were injected either by multiple loads or with a single load which is a common practice during ICSI with experienced embryologists. All eggs injected (n=34) showed a fluorescent signal when imaged, indicating that the carried over solution remained in the injection pipette after crossing to the injection drop (Fig.4. 16). Figure 4. 17 represents various experiments conducted for developing a protocol for the co-injection technique.

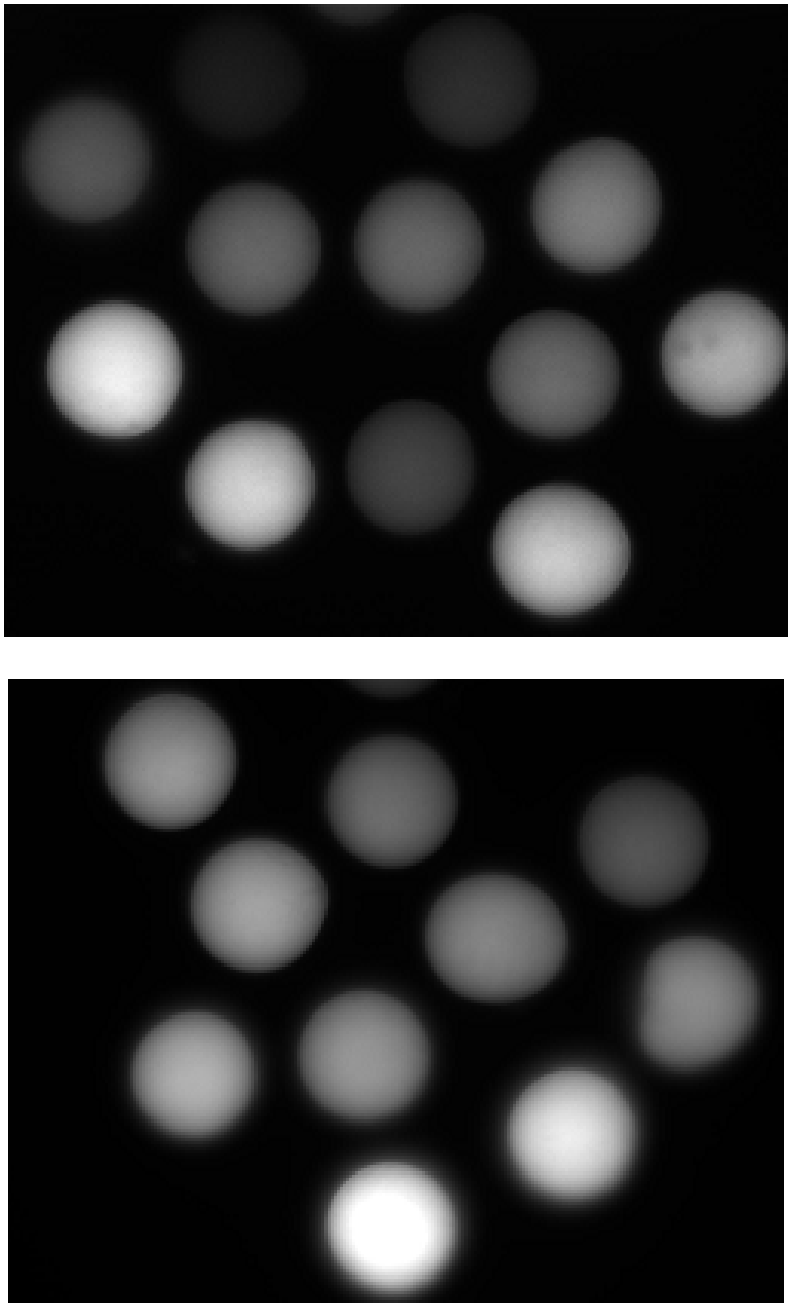

Figure 4.16 Fluorescent images following a protocol for co-injecting sperm and hPLCζ using the mouse ICSI pipette.

The top image is for mouse eggs injected with the fluorescent indicator using a high pressure injection system and the pipette used for that system. The bottom image is for eggs post co-injection of sperm and hPLCζ after carry over from a drop containing 10%PVP using a mouse ICSI pipette. Both injection materials contained the same fluorescent indicator.

To examine if the results would be consistent when PLCζ protein is added to the PVP solution, a stock that had an initial concentration of 0.06mg/ml was used in the next set of experiments. The protein was diluted down to 0.01mg/ml in KCL-Hepes with 10%PVP and the mix was co-incubated for 30minutes on an ice block. The dish was setup as mentioned previously, when the protein drop was examined under 20X it appeared that there was aggregate formation (Fig.4.18). A brief spin in the bench top centrifuge to assess if the aggregates would re-dissolve in the solution proved to be of no benefit. On the contrary, it promoted an increase in the apparent size of the aggregates. I carried on with the experiment to assess if the aggregate formation has caused any effect on protein function. ICSI was performed with the use of the mouse ICSI pipette, the protein mix that had the smaller aggregate size was used.

In the first run for this experiment, due to the difficult control over the advance of the injection material within the injection pipette, each pipette load was used to inject up to 3 eggs. A total of 12 eggs were injected, 9 survived (75%), 6 eggs showed Ca^{2+} oscillations. However, the frequency amongst the six eggs was variable, ranging from a very high frequency to a single initial spike. All eggs released a second polar body, in the high frequency pattern cell fragmentation was noticed. The variability of the oscillation frequency was very likely to be caused by the unequal amounts being injected into the eggs. Therefore, to have control over the amount of media injected into each egg, a separate load was taken for every single egg. A total of 25 eggs were injected in this second run, first, 12 eggs were injected, 9 survived (75%) and 8 eggs oscillated when imaged. The next group of this second run included 13 eggs, 10 survived (76.9%) and 9 oscillated. With this method of injection the variability previously seen in the oscillation frequency was reduced (Fig.4. 19). Results were indicating that the method of co-injecting the protein and sperm is a valid technique. It had the advantage that it would not need any extra training sessions for embryologists as it is the current protocol in use. However, the aggregates that were seen in the media needs to be further analysed in terms of components and if any protein activity was lost due to this effect. Could PVP be replaced by any other molecule that would be fit for purpose, slowing the sperm and being inert to PLCζ protein? This enquiry has been picked up as a separate project in our laboratory.

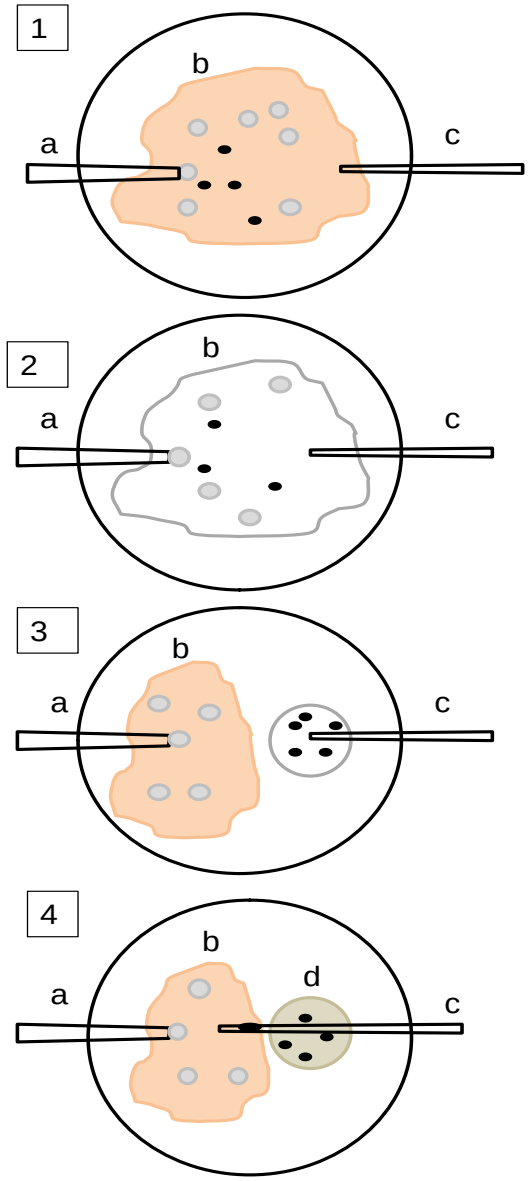

Figure 4.17 Schematic diagrams for the various protocols tested for the co-injection technique.

1, A single drop of M2 containing mouse eggs and sperm with hPLCζ at the required concentration. **2**, A trial for using KCL-Hepes as an injection medium for the co-injection protocol. **3**, Using a separate drop for the co-injection technique (KCL-Hepes with hPLCζ and sperm), to be carried over to the injection drop using the mouse ICSI pipette. **4**, Supplementation of the KCL-Hepes drop with 10%PVP on top of hPLCζ and sperm.

a, Egg holding pipette. **b**, Injection medium (M2, KCL-Hepes in 2). **c**, Mouse ICSI pipette. **d** Sperm suspension drop with 10%PVP and hPLCζ.

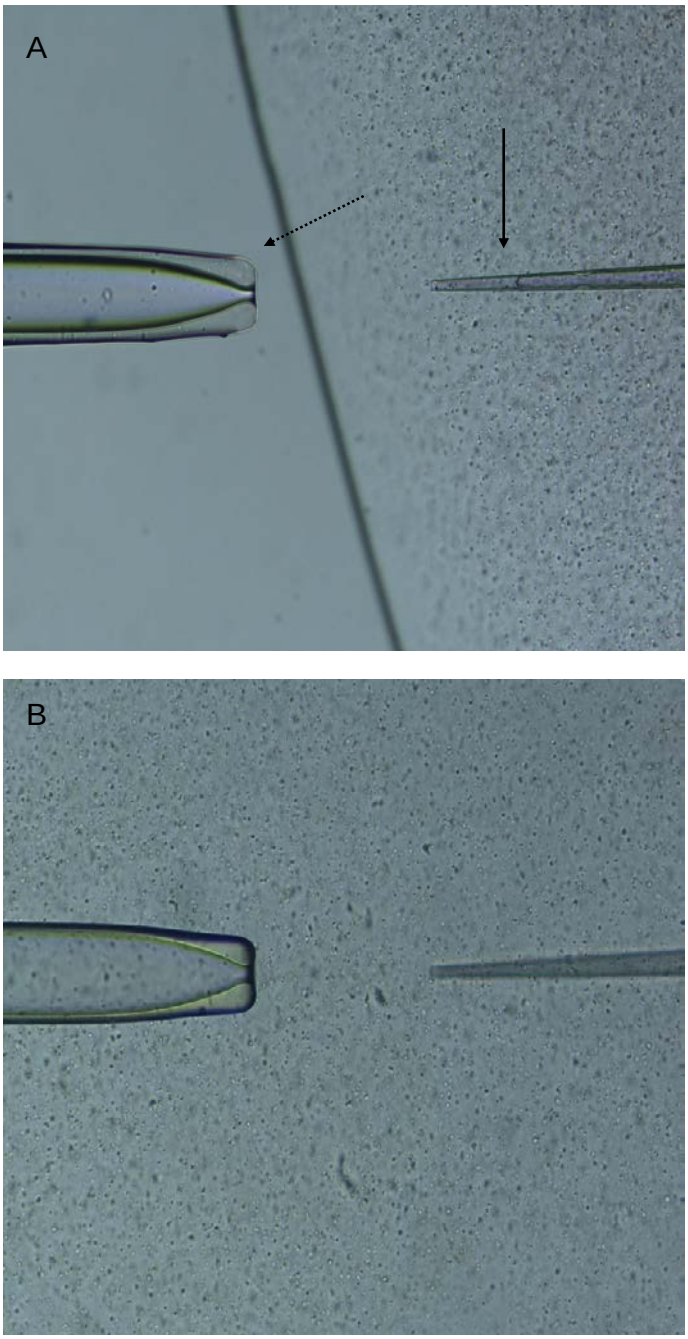

Figure 4.18 DIC images for aggregates formed when hPLCζ was mixed with 10%PVP.

Images of the KCL-Hepes drop with 10%PVP and a final 0.01mg/ml of hPLCζ protein. The dashed arrow points to the egg holding pipette held on the edge of the drop, while the block arrow shows the injection pipette with the some injection media taken inside. The surrounding medium has specs dispersed within the whole drop.

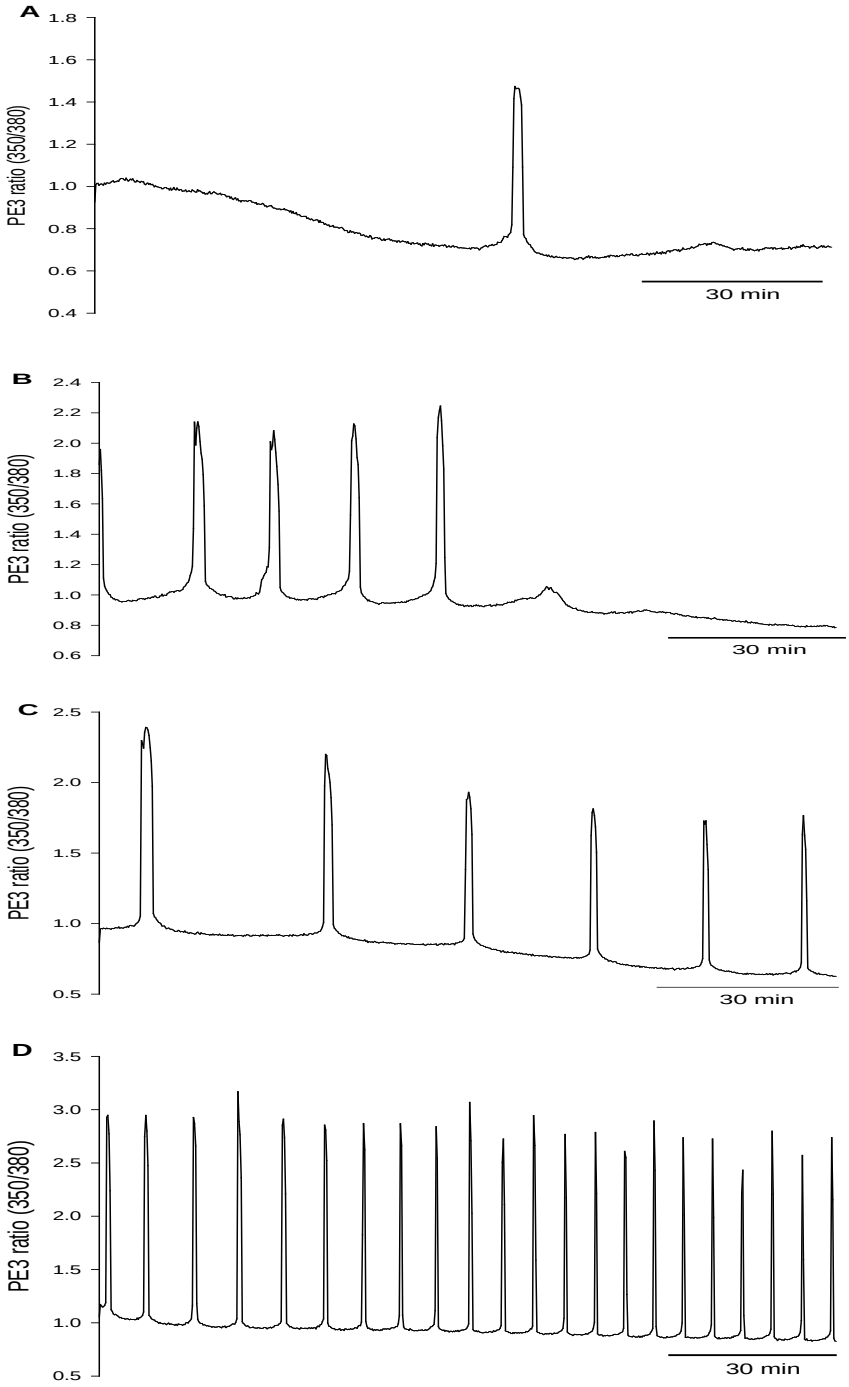

Figure 4.19 Representative traces for Ca^{2+} oscillations detected following a co-injection protocol for sperm and hPLCζ.

A, One egg had only one spike after a latent period. **B**, Ca^{2+} oscillations started regularly but had a premature stop. **C**, A response detected that resembles the physiological Ca^{2+} oscillation frequency pattern. **D**, A trace representing traces form eggs that had a heavy frequency of oscillations.

4.4 Discussion

Gamete micromanipulation was introduced into the IVF field to overcome failures seen in conventional IVF treatments, with ICSI procedure having the highest degree of invasiveness (Ebner et al., 2001). Even with all the advances in protocol development, cases of fertilisation failure are still documented post-ICSI. The failure rates following ICSI were mainly caused to activation failure (Tesarik and Sousa, 1994, Nasr-Esfahani et al., 2010). A growing interest within IVF clinics for the use of artificial activation protocols has occurred to try and solve cases with fertilisation failure. With the sperm factor currently widely agreed to be the PLCζ protein, its use during ICSI cycles to rescue activation failure has received increasing attention.

This section of my study was to focus on developing a reproducible methodology to co-inject PLCζ protein during ICSI and to further asses the response of human eggs to a PLCζ protein injection. I had the advantage that our lab has made the active recombinant human protein (hPLCζ); the protein made had a NusA tagged to its C terminus (Nomikos et al., 2013). NusA as a fusion protein has a dual function of maintaining stability and solubility of recombinant proteins (De Marco et al., 2004). In unpublished data the PLCζ protein was injected into mouse eggs without the NusA tag, there would be a dramatic loss of the protein's activity seen as diminished Ca^{2+} oscillation frequency. That indicates the importance of the fusion tag for protein function. Previous studies published by our group have shown that a microinjection of hPLCζ protein could support blastocyct development in mouse eggs (Yu et al., 2012). Moreover, it has been shown that microinjecting NusA fusion tag on its own along with a live sperm did not have any detrimental effects on the blastocyst development rate in mouse eggs (Sanusi et al., 2015). In my results I have injected hPLCζ protein into human eggs that have failed an IVF procedure. The same concentration was injected in mouse eggs as well as human eggs (0.015µg/ml), the main difference detected between the eggs that have oscillated in both groups was in the frequency of Ca^{2+} oscillation. A general perception from the results is that the mouse eggs had a higher frequency of oscillations. The data presented is supported by original data with regard to the Ca^{2+} releasing ability of mPLCζ in comparison to hPLCζ in mouse eggs, when hPLCζ had a higher potency in mouse eggs (Cox et al., 2002). The difference seen in response to PLCζ of different species could be explained by a number of possibilities. First, is that the human egg is about five times bigger in volume than the mouse eggs. The human egg has a diameter of

around 120µm without the zona pellucida, while the mouse egg's diameter is approximately 70µm. Therefore, a dilution factor effect due to size difference could explain a weaker response in human eggs when compared to mouse eggs. The extra volume in the cytoplasm of human egg over the mouse egg would require an extra amount of protein to trigger a similar response to that of the mouse egg. Differences between species with human and mouse eggs could also be due to different sensitivities to PLCζ protein. In the case of hamster eggs for an instance, it was found that, compared to mouse eggs, their response to sperm extract showed a lower sensitivity despite the egg being the same size (Parrington et al., 1996).

The other main difference that exists between both groups of eggs injected is that mouse eggs were freshly ovulated when used, while human eggs were Day 1 (over 24hrs post collection). Post ICSI/IVF procedure human eggs are checked for pronuclei formation at about 17hrs later, and then for my experiments the eggs had were transported in a temperature controlled flask with a drive of about 2hrs. So the transportation could have affected the eggs as well. The age factor could have possibly caused the inefficient response to hPLCζ microinjection. To test for a real difference in sensitivity, that would require freshly ovulated eggs from both species. The limited availability of donated human eggs for research would make that quite problematic. However, it would be interesting to determine the effectiveness of hPLCζ in supporting development up to blastocyst stage in freshly ovulated eggs and compare that to conventional ICSI/IVF alone.

The degree of oocyte maturation has a fundamental role in the egg's ability to release Ca^{2+} , the Ca^{2+} release mechanism go through a series of changes throughout this process (Mehlmann and Kline, 1994, Carroll et al., 1994). Moreover, the given ability to any egg to respond by the series of long lasting Ca^{2+} oscillations is entirely dependent on a successful journey through maturation (Jones et al., 1995). The response that I have seen in immature oocytes (MI, GV) could therefore be explained by the poor cytoplasmic maturation. Further support to this is that it has been shown that in immature mouse eggs, the response from sperm fusion results in either a single Ca^{2+} transient or maximally three transients (Jones et al., 1995). The only GV that had a Ca^{2+} oscillation response similar to that of fertilisation was the one that matured into an MII oocyte. Whether it was a cause or a response I cannot comment on, as I have no evidence for any of those explanations. The experiments carried out on human eggs that have failed to fertilise were subject to a

number of limitations. Previous handling/transportation as well as the number of eggs included, that made the conclusions from results in need of further support from further studies.

In the remaining part of this section of results I went on into testing whether the activation capacity of hPLCζ protein would be preserved via freeze-drying, as it is a common method used to preserve function of biologically active material after transformation into a solid form. After injecting mouse eggs with the same concentration of hPLCζ protein (0.01mg/ml) that has been freeze-dried, a significant difference was seen between groups indicating that the protein that was reconstituted and kept overnight at $4C^0$ had the highest activity preservation. This result is supportive of the idea that hPLCζ protein matures over a period of time to gain its maximum ability to hydrolyse PIP_2. However, when protein storage was examined, I injected a range of protein concentrations into mouse eggs again for 3 different storage spots. It was apparent that the concentration of protein used has an effects on the response elicited for the storage method. The freeze-dried batch kept at 4^0C seems to have an overall persistence of activity as compared to the other 2 storing conditions (-80^oC, at room temperature). Basically there are two types of protein stability which are important, the first is during freeze-drying process which proved to be maintained for hPLCζ protein. The second is stability preservation during storage. Constantino et al showed how some proteins can be denatured by forming aggregate during storage after freeze-drying due to the prescence of moisture (Roy and Gupta, 2004). Another explanation could be aggregate formation due to formation of covalent non-disulphide bonds, as shown in tetanus toxoid after freeze-drying. A proposed preventive strategy to see if the relative deterioration in hPLCζ protein function is due to these proposed mechanisms is to add a preservative, or lower the pH during freeze-drying process. Due to time limitation further work on this issue was put on hold and to be resumed later within the laboratory.

The hPLCζ protein currently is microinjected into mouse eggs and human eggs, as a second injection subsequent to ICSI, using a high pressure injection device which is not available in IVF clinics. To design a practical method that could easily be reproduced by embryologists, I first tested a strategy that includes a second injection that would not require a high pressure injection. This consisted of a bevelled pipette that was made in the laboratory and that could be used with low

pressure equipment found in IVF clinics. However, the issue with this method was the diffusion of material after carrying the pipette across to the injection medium. This problem is also seen with conventional ICSI pipettes, so it seem reasonable to juts use a standard (mouse) ICSI pipette since this could, at least, be used for a single ICSI and PLCζ injection protocol.

With the change back to the ICSI pipette, the first hurdle was the low survival rate post injection. This was due to the initial Ca^{2+} transient that was too big for the egg to handle. The injection itself creates a Ca^{2+} increase within the cytoplasm and with hPLCζ protein being in the injected material as well, it leads to a potentiated $Ca2^{+}$ rise that caused a significant egg loss post injection. Egg damage and loss was also the result with a co-injection protocol with the injection media either with Ca^{2+} or with no Ca^{2+} or with a low level of Ca^{2+}. The idea that Ca^{2+} absence from the injection media leads to cell lysis is supported by an early report by Steinhardt RA et al. They showed that in unfertilised sea urchins a micropipette induced membrane puncture requires a minimum level of 300µM extracellular Ca^{2+} in order for resealing to occur. These studies indicated that a Ca^{2+} dependant mechanism for resealing might include vesicle delivery, docking and fusion, in a similar fashion to what occurs during exocytosis of neurotransmitters (Steinhardt et al., 1994). Hence, failures may occur by excluding Ca^{2+} within the injection media to improve the survival rate for eggs post ICSI.

I tested the effect of increasing the osmolarity of the injection medium and there was a perceptible increase in membrane rigidity that could be explained by the reduction of membrane fluidity due to hyper-osmotic stress (Kaufman and Surani, 1974). The increase in osmolarity did not have any beneficial effect on the egg survival rate post ICSI. The return to the original configuration of conventional ICSI had a more successful result. The protocol included supplementing the sperm drop that originally had 5-10% PVP with the required dose of hPLCζ protein. Carrying over protein to the injection media was controllable given the viscosity of PVP and so the triggering of Ca^{2+} oscillations could be recorded. The survival rate of eggs post injection was almost restored to usual levels post ICSI for my personal experience (80-90%) However, there were noticeable aggregates seen within the PVP drop which could explain the variability in the frequency of oscillations detected. It is possible that aggregation occurs on smaller scale when hPLCζ is diluted in KCl Hepes medium and that this explains some variability in the response of human

eggs to hPLCζ seen in the above section. A further exploration of the composition of the aggregates formed would be informative. The possibility of a substitute for PVP, which may be the main cause behind aggregate formation, might also be considered. Furthermore, a trail run for assessing mouse embryo developmental capability post ICSI using the co-injection technique post, resolving the aggregation issue, could bring the use of hPLCζ protein in IVF clinics a step closer.

Chapter 5

HyPer Probe, A Novel Application in Mouse Eggs

5 HyPer Probe, A Novel Application in Mouse Eggs

5.1 Introduction

Mitochondria are unique intracellular organelles in that they have their own DNA which allows them to replicate independently of the hosting cell (Ernster and Schatz, 1981). They are the major providers of the energy demand of the cell in the form of ATP, they are the main site for the citric acid cycle, fatty acid oxidation and oxidative phosphorylation (Ernster and Schatz, 1981).In offspring all genetic material come from maternal and paternal DNA, each side providing and equal contribution. One exception is that of the mitochondria, all mitochondria come are derived from the maternal side (Shoubridge, 2000). More than the supply of ATP to the cell, in eggs they play a critical role in regulating the Ca^{2+} changes that occur during fertilization in ascidians (Dumollard et al., 2003) and mouse eggs (Dumollard et al., 2004). It seems that there is a bidirectional interaction between Ca^{2+} signaling during fertilization and the mitochondria. It has been found that the Ca^{2+} increases occurring in an oscillatory pattern are necessary to trigger the turnover of ATP from mitochondria (Campbell and Swann, 2006). This dynamic interaction between the Ca^{2+} signaling during fertilization and the mitochondria opened a research area to study mechanisms of interactions and how that subsequently affects the outcome of the fertilization process. 3 main areas of interest have been identified, first is the buffering capacity of mitochondria for the changing cytosolic Ca^{2+}, ATP turnover and the production of reactive oxygen species (ROS). Mitochondria is one of the major sources of ROS, Ca^{2+} is thought to be able to stimulate the oxidative phosphorylation process (García-Pérez et al., 2002). Available data suggests that during fertilization there is an increasing demand on the antioxidant defense system, as the level of glutathione (an antioxidant defense enzyme) was down by about 45% by the end of the Ca^{2+} oscillations triggered at fertilization (Luberda, 2005). However, there is no direct evidence of an increase of ROS available. Perturbation of the antioxidant defense mechanism or an increase of the levels of ROS can negatively impact embryo development (Johnson and Nasr-Esfahani, 1994, Harvey et al., 2002, Guérin et al., 2001). Moreover, within every cell hydrogen peroxide a short living molecule and a member of the ROS, has a wide impact on a spectrum of biological processes. Hydrogen peroxide (H_2O_2) is considered an important second messenger within the cell and the germ cell line is of no exception. ROS are known

to have a detrimental effect on the cell viability if levels rise beyond the ability of the cell to scavenge the excess. However, other than having the negative impact, they are vital for certain essential enzymatic activities within the cell and have a role as cofactors for a number of transcriptional factors (Dröge, 2002). Reports available on the estimation of ROS production *in vitro* are mostly dependant on indicators that are controversial in their use. The dicholorfluorescein (DCF) indicators are crticised for their non-specific reaction with ROS as they interact with nitrogen species as well (Crow, 1997). Moreover, available data suggest that the indicator itself during exposure to light produces ROS (Belousov et al., 2006). Making results from the use of the DCF questionable, as to how much of the signal detected is a genuine production of the cell and not an indicator contribution?

Studying H_2O_2 (ROS) has proven to be challenging, however, a modern genetically encoded fluorescent biosensor, HyPer has recently been reported to be a selective H_2O_2 indicator in living somatic cells. The indicator has promising results in regard to its selectivity and specificity to H_2O_2 (Belousov et al., 2006). The basis behind the probe is the OxyR regulatory domain of *Escherichia coli,* the domain is specifically sensitive to H_2O_2. To develop a fluorescent probe, the group inserted a circular permuted yellow fluorescent protein (cpYFP) into the regulatory domain. The signal is a result of a confirmatory change due to a transformation from the reduced to the oxidised form by the exposure to H_2O_2. The oxidisation allows formation of a disulfide bond between two cysteine residues (Belousov et al., 2006). Moreover the probe is a ratio-metric indicator, giving an advantage of the signal being independent of the amount of probe available.

5.2 Aim

With all data available around the new probe for H_2O_2 I wanted to find out if this sensor could be used to assess the intracellular *in vitro* redox state represented by H_2O_2 in mouse eggs. The ultimate aim was to identify an explanation to the long term effect of patterns of Ca^{2+} oscillation during fertilisation on later events of embryo development. As to how an early phenomenon influences embryo development days after it has ceased. The main hypothesis is that different patterns of Ca^{2+} oscillation during fertilisation cause variability in the levels of ROS produced which eventually influence embryo development.

5.3 Results

5.3.1 Characterisation of the HyPer probe signal after injection into mouse eggs.

HyPer, is a genetically developed biosensor that is claimed to enable to follow the dynamic changes of H_2O_2 in living cells. There is no report for the use of the HyPer probe in the context of any eggs and the effect of fertilisation or parthenogenetic activation on the oxidative system. In this section we aimed to investigate the use of this probe in mouse eggs, as oxidative stress is a possible explanation for an abnormal course of development in embryos. However, quantifying the levels of H_2O_2 as a measure of ROS has proven to be a challenge. This part of work reports the use of the HyPer probe in mouse eggs which is a novel application. To enable this, signal detection and its separation from other signals that could be used in conjunction was essential. The experiments in this Chapter are designed to examine if detection of fluorescence signal from HyPer probe is possible after it is micro-injected into mouse eggs.

The HyPer protein was initially microinjected into mature mouse eggs and a fluorescence ratio signal recorded. Figure 5.1 A, B shows that the fluorescence signal ratio was measurable and remained constant for the duration of imaging, the imaging was done with two different exposure duration (100ms, 500ms) and both showed similar difference in results. With the use of this probe the intention was to be able to use it for prolonged durations extending to hours, in parallel to measurements of Ca^{2+} oscillations. The next experiment was to determine whether the HyPer signal can last throughout that length of an experiment without compartmentalisation or bleaching of the probe. For this purpose MII mouse eggs were micro-injected with the HyPer probe and incubated for 4 hours in a drop of M2 covered by mineral oil. Then fluorescence ratio from the eggs was later recorded and continued for 2 hours. Although there was sometimes a drift in the signal, no substantial reduction in the signal was detected and a comparable ratio level and noise level was seen to that of freshly injected eggs (Fig. 5. 1C). Figure 5. 2 reveals the photo-bleaching effect for various exposure duration (A, B) and a prolonged exposure for 4hours after microinjection (C) for the single wavelengths of the probe.

5.3.2 Does HyPer probe respond to changes of H_2O_2 in mouse eggs?

The HyPer probe is proposed to rely on a conformational change in triggering a fluorescent signal change. A disulfide bond forms between two Cys residues due to the oxidisation of either one of them. This conformational change will cause the cYFP to shift its fluorescent spectrum in relation to the amount of H_2O_2 available (Belousov et al., 2006). To find out if HyPer could be utilised in mouse eggs, MII mouse eggs were microinjected with the H_2O_2 sensor and placed on a preheated dish on the fluorescence imaging system. Measurement of the fluorescence before and after the addition of H_2O_2 was done. A range of H_2O_2 concentration was used to measure the response (100µM 1mM and 10mM). The minimal response of the probe was seen with a concentration of 100µM H_2O_2 (Fig.5. 3A, B, C). An increase of fluorescence excitation ratio was seen when the concentration of H_2O_2 was increased. The signal increase indicated an oxidation event of the probe. However, with an increase in the duration of imaging to beyond a full hour the signal did not recover to the pre H_2O_2 baseline. It has been reported that the probe oxidisation outlasts the peroxide removal by the antioxidant system within the cell which means reversal of the probe oxidation status is rate limiting (Bilan et al., 2013, Lim et al., 2014). Another explanation is that the probe relies on cellular disulfide reductase activity to bring the probe back to its reduced state. Any perturbation in this scavenging mechanism will make the probe no longer available for another cycle of oxidation in vitro. All eggs post imaging were looking healthy except for those exposed to the highest concentration of H_2O_2 used which seemed to be swollen. Once the eggs were swollen, they did not recover and eventually died. Despite these effects at high concentration, these data do show that the HyPer probe can at least respond to a large change in H_2O_2 concentration.

5.3.2.1 Does DTT affect the HyPer probe signal?

The signals from the HyPer probe are caused by a disulphide bond forming and breaking between two active SH groups. Dithiothreitol (DTT) is widely known to be a preventive agent for formation of intra-molecular and inter-molecular disulfide bonds. It was prepared as 1M stock solution in water and kept in -20^{o}C until further use. DTT was mixed with the probe in order to maximise its potential to be oxidised and to aid in the reduction post oxidisation during the addition of the peroxide (H_2O_2). Additionally, to see the effect of prior reduction of the HyPer probe, it was co-

microinjected into mouse eggs with DTT. A final concentration of 1mM was made when added to the HyPer probe and incubated for about 10 minutes before further use. The recording shown in (Fig.5. 4) shows that the fluorescence ratio, and the response to H_2O_2, was similar to an egg injected with HyPer probe without DTT. These data suggest that prior reduction of the HyPer probe does not substantially effects its behavior in eggs.

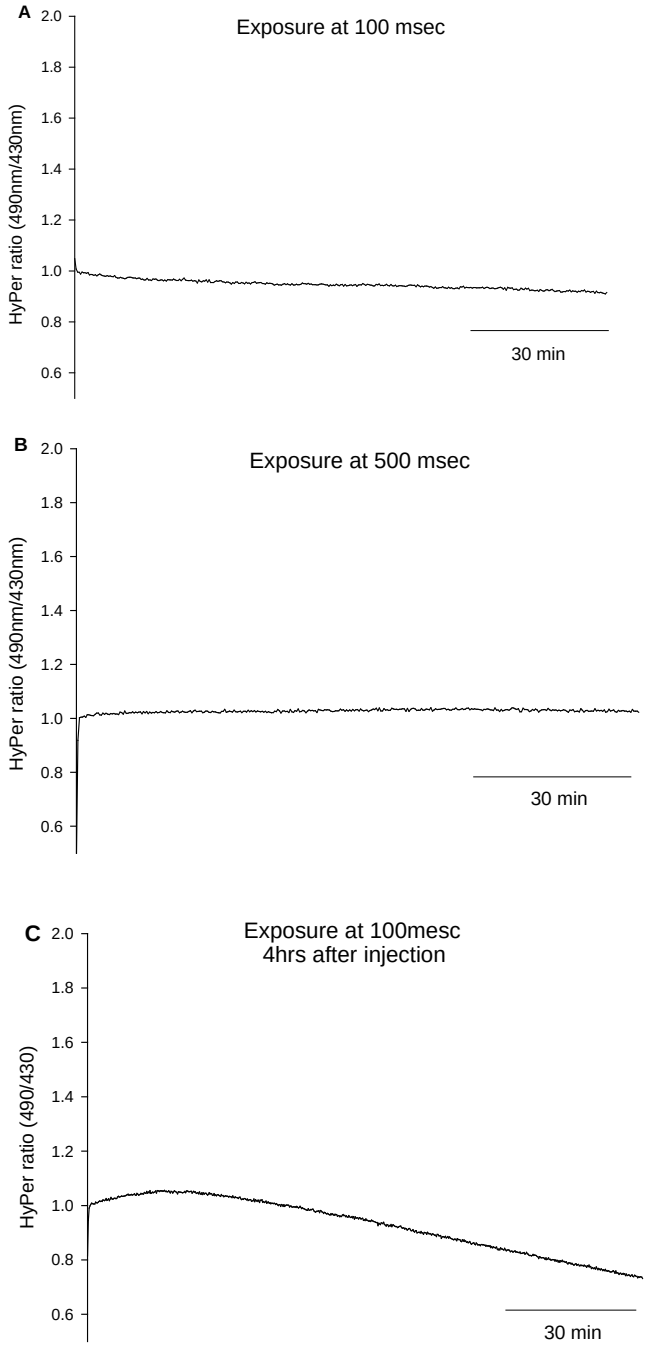

Figure 5.1 HyPer fluorescent signal ratio detection in mouse eggs.

The traces are representative of various excitation protocols to HyPer probe after micro-injection into mouse eggs. A, HyPer probe signal during an exposure with 100ms exposure (n= 16). B, An exposure of 500ms has no negative effect on the signal detected (n= 12). C, A prolonged exposure at 100ms exposure shows no substantial photo bleaching effect on the probe (n= 17).

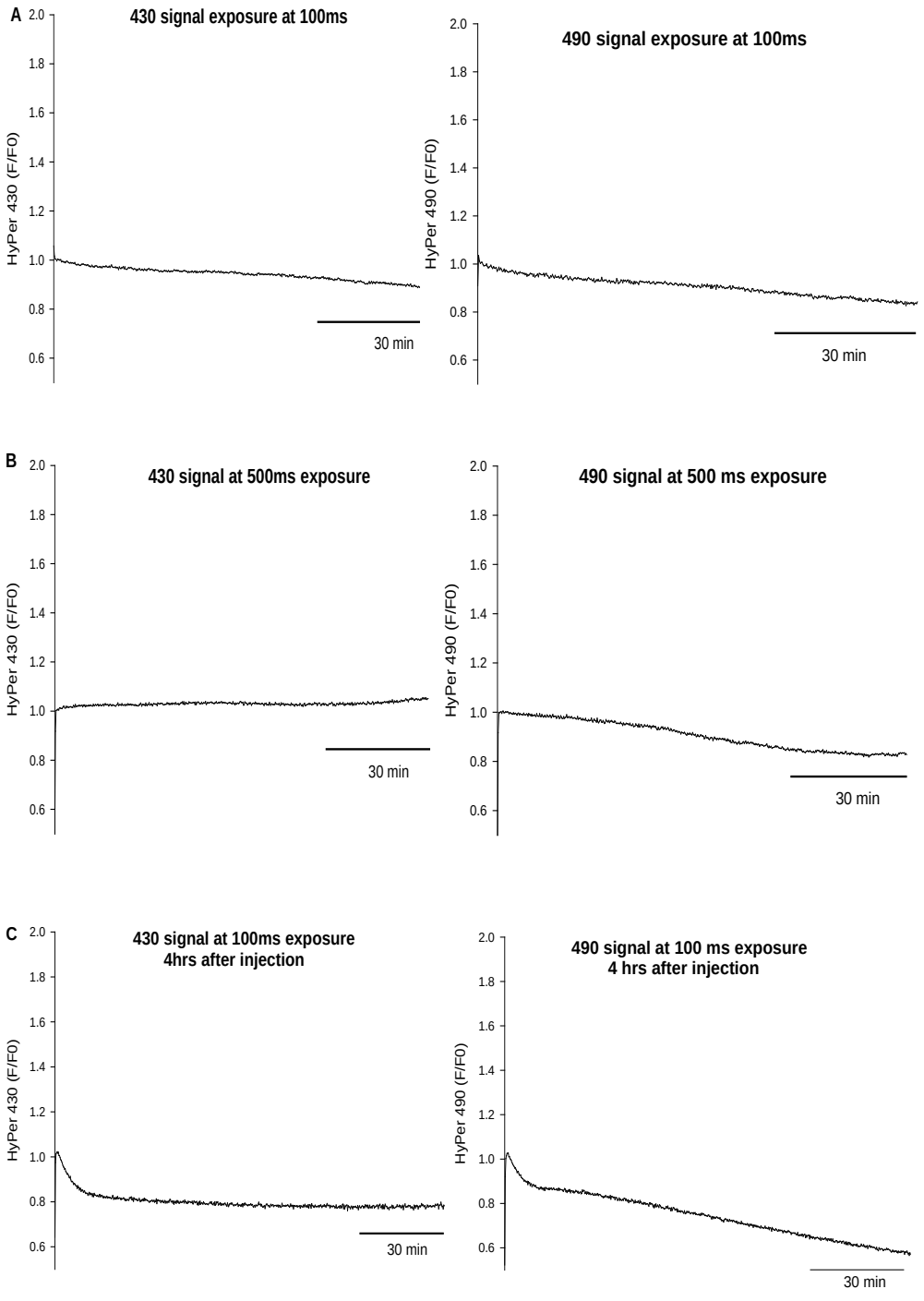

Figure 5.2 Fluorescent signals from the single wavelengths of the HyPer probe under various conditions.

Traces representing the effect of photo bleaching on the single emission wavelengths for the HyPer probe. **A,** Effect of 100ms exposure on 490, 430 signals. **B,** 490, 430 signal Exposure to 500ms. **C,** The effect of 100ms exposure after 4hrs from the injection procedure.

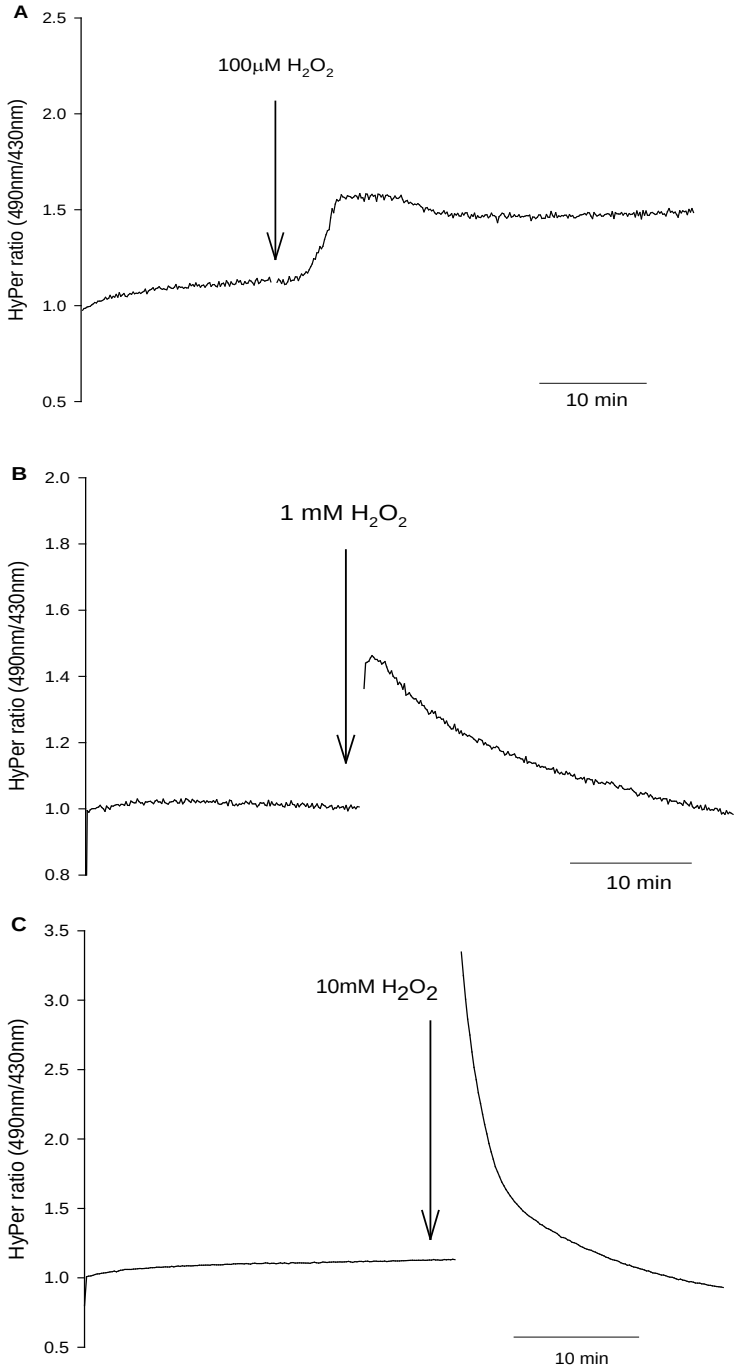

Figure 5.3 Hyper probe response after micro-injection into mouse eggs with the addition of various H_2O_2 concentrations

A, HyPer probe signal ratio pre and post addition of 100μM of H2O2 (n= 23). **B** representative trace for the effect of 1mM H_2O_2 on the HyPer probe fluorescent signal (n= 20). **C,** A tenfold increase in the concentration of H_2O_2 effect on HyPer signal detected in 18 mouse eggs. Arrows indicate to the time point where H_2O_2 was added.

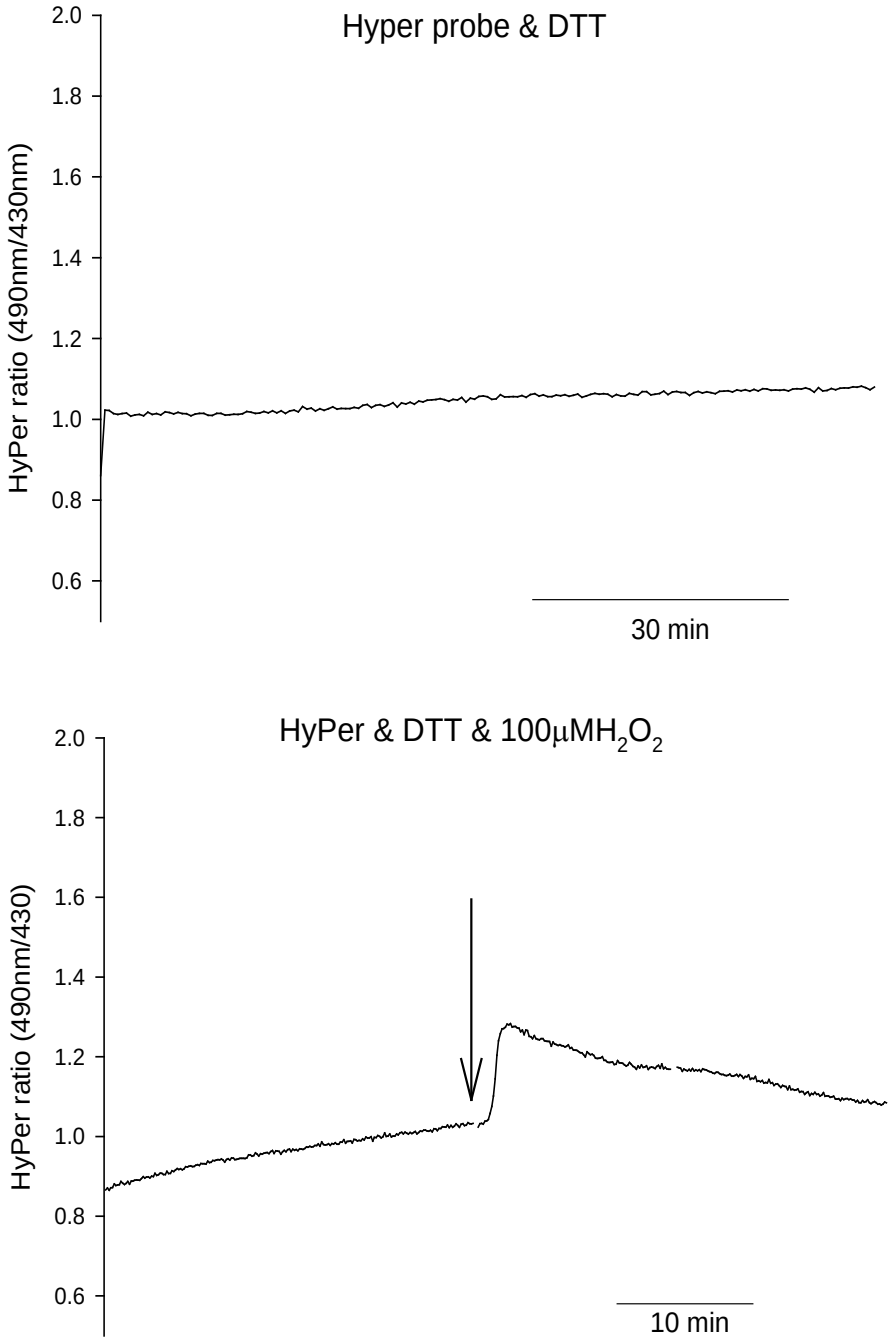

Figure 5.4 Hyper probe signal with DTT.

Traces representing the effect of the co-injection of DTT with the HyPer probe in mouse eggs. **Top trace,** the signal detected for HyPer probe shows no change in comparison to traces representing HyPer alone (n= 24). **Bottom trace,** HyPer signal and DTT response to the addition of 100μM of H_2O_2, arrow indicates the point of addition (n= 22).

5.3.3 The relationship between signals detected due to cytosolic Ca^{2+} change and the HyPer probe signal.

The main aim of this chapter was to investigate a potential existence of a change in the H_2O_2 levels in parallel to the cytosolic increase of Ca^{2+} during parthenogenetic activation or at fertilisation. A change in H_2O_2 levels could be generated by mitochondria and could override the ability of the regulatory system to scavenge the excess production. The production of H_2O_2 could be considered as a signal for a number of intracellular biological functions, be it could also have a detrimental effect. In the latter case embryo developmental arrest could be due to the early events in mouse eggs associated with Ca^{2+} signals.

5.3.3.1 No interference between HyPer probe signal and Ca^{2+} fluorescent indicators.

Another factor in determining the possible use of HyPer probe for the study was the need for its signal to be distinguishable from that of Ca^{2+} indicators. Here, a direct comparison of both indicators is made. Under my loading and illumination conditions, I have found that both indicators have permitted a long term measurement. Rhod-dextran as a Ca^{2+} indicator can be used at an excitation and emission wavelengths (ex. 556nm- em. 677nm) that would not conflict with the two peak excitations of 500nm and 420nm of the HyPer probe and its one peak emission at 516nm. Figure 5.5 demonstrates that both indicators have been recorded for an extended duration of about 9 hrs, a steady and reliable fluorescence signal was verified for both indicators.

5.3.3.2 Hyper probe does respond to cytosolic Ca^{2+} changes induced by Ca^{2+} Ionophore Ionomycin.

Cytosolic Ca^{2+} levels in mouse eggs could be manipulated either in a physiological manner or by inducing a parthenogenetic activation. The form of alteration in terms of level and frequency can differ between methods used. Here we made a comparison between the effects of Ca^{2+} transients induced by Ca^{2+} ionophore (5µM Ionomycin) along with other agents, PLCζ and IVF (Fig.5. 5, Fig.5. 6 and Fig.5. 7). The MII mouse eggs were injected with the HyPer probe and Rhod-dextran and after a period of recovery of about 10 minutes the eggs were placed on the fluorescence imaging system. In all activation protocols a signal was obtained before adding the stimulus. Figure 5. 5(A, B, C) shows the first experiment in this

series where a final concentration of 5µM ionomycin in the dish induced a predicted rise in the Rhod-dex signal. Alongside this there was a rise in the fluorescence ratio of the signal of HyPer probe suggesting a possible parallel rise in the level of H_2O_2. Whereas a Ca^{2+} rise induced by IVF or PLCζ injection fails to induce a change in the HyPer probe signal similar to that induced by ionomycin. We observed that there was a sharp rise in the fluorescence signal of the probe concomitant with the addition of the ionophore. In figure 5.6 A, B, C it can be seen that the fluorescent signal of HyPer showed some signs of an increase with the addition of 0.5µM ionmycin but not during the initial addition of thapsigargin (endoplasmic reticulum Ca^{2+} ATPase inhibitor).

Since there was an indication that Ca^{2+} possibly influences the level of ROS as represented by H_2O_2 within the egg cytoplasm, examining the effect of other Ca^{2+} modulating protocols was necessary. IVF and micro-injection of PLCζ protein were the two protocols examined. The IVF protocol as described elsewhere was tested first. For all experiments in this instance, a trace for the signal of both probes was obtained as a reference for any upcoming change after adding sperm to the media. In the case of PLCζ protein micro-injection, it was not always the case that a long trace was captured prior to the start of the Ca^{2+} oscillations. The action of the PLCζ protein is more rapid (within minutes), however, in some traces it was manageable. A similar response to the ionophore addition was not detected when IVF or PLCζ were used as a method of inducing cytosolic Ca^{2+} changes and egg activation. The Ca^{2+} oscillation associated fluorescence signal of rhod-dextran were associated with a gradual increase in the HyPer probe signal (Fig.5. 7 a, b). However, this signal was not related to the Ca^{2+} changes in eggs because the gradual signal increase started before the onset of Ca^{2+} oscillations. In addition when the signal from eggs that were not fertilized by sperm were analysed, the same gradual rise in HyPer probe ratio was also observed. The gradual increase of the signal was that of equilibration as time was needed for the probe to reach plateau for the response. The same observation was documented for the PLCζ protein micro-injection group. Hence the only HyPer probe response detected seemed to be with ionomycin. In one experiment the Ca^{2+} oscillations were set off by PLCζ where no HyPer probe response was detected. After a while, an addition of 5µM ionomycin was made to the oscillating eggs, and then a HyPer probe response was detected (Fig.5 7 C).The results were consistent throughout 3 separate runs for each group.

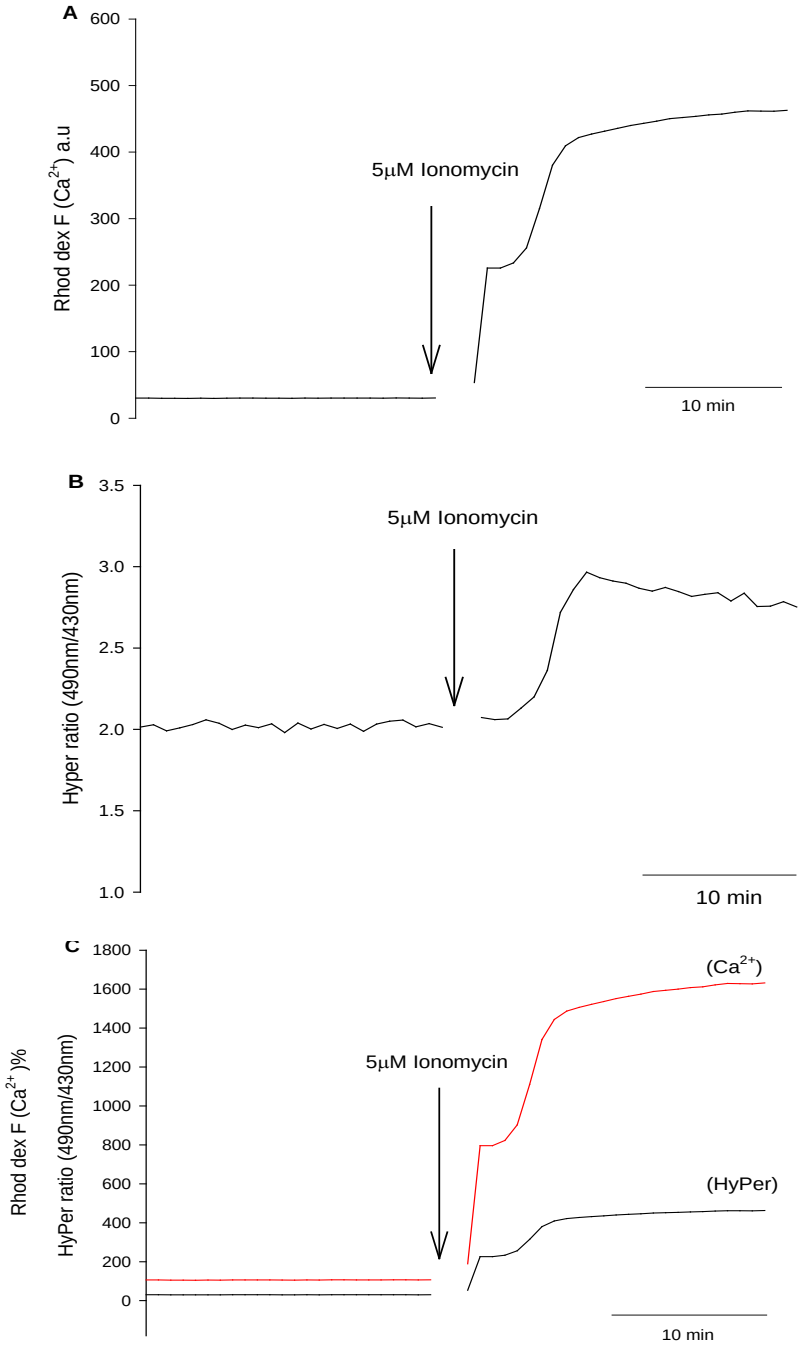

Figure 5.5 HyPer probe and Ca^{2+} indicator signal interaction.

A, A sample trace for Rhod-dex (Ca^{2+} indicator) in response to the addition of 5µM ionomycin (arrow). **B,** Hyper probe signal in response to the addition of the ionophore, HyPer and Ca^{2+} signals were obtained from the same egg (n= 25). **C,** Signal overlay for both probes (Hyper and Ca^{2+}).

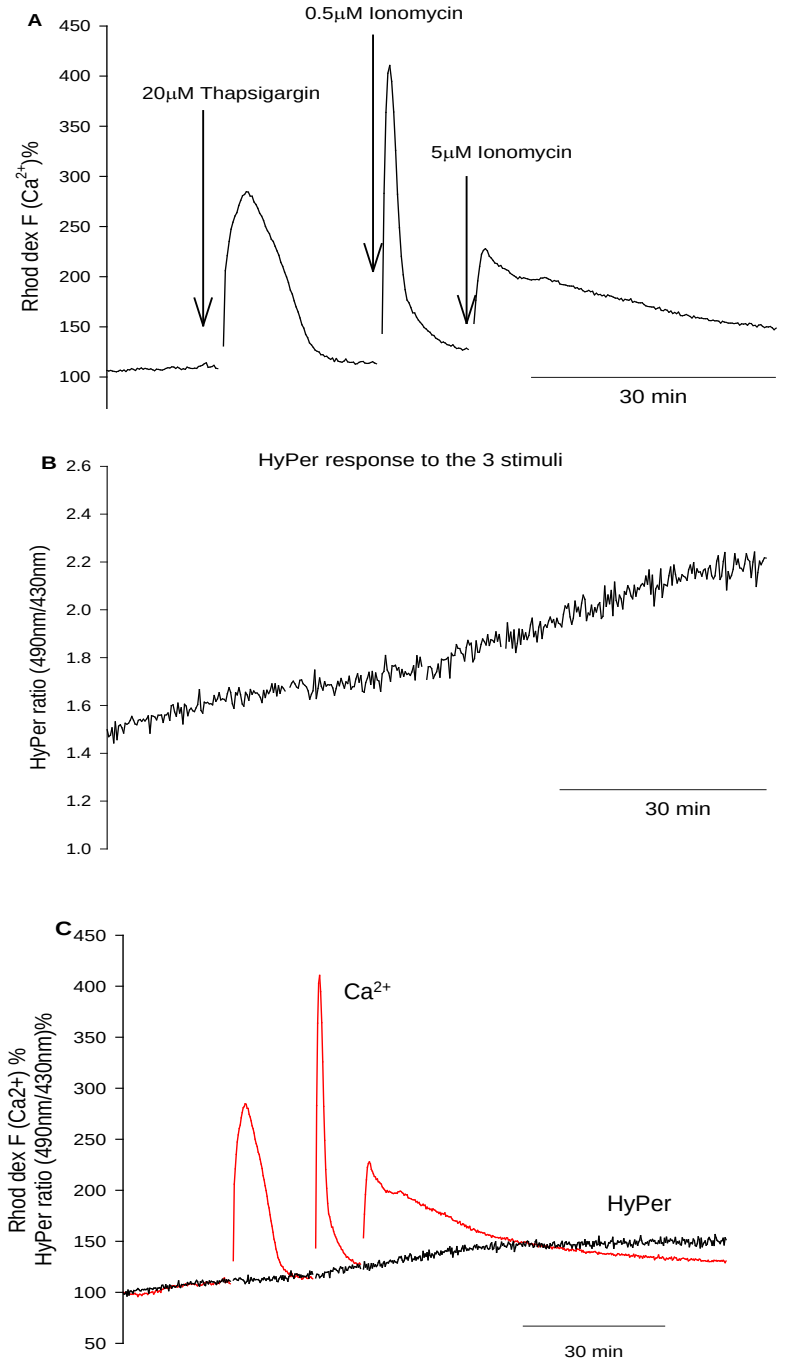

Figure 5.6 The effect of thapsigargin and two different concentrations of ionomycin on the HyPer probe signal.

After a co-injection of Hyper probe and Ca^{2+} indicator into mouse eggs signal of both were detected. **A,** The response of the Ca^{2+} indicator to the addition of 20µM thapsigargin followed by a 0.5µM ionomycin then 5µM ionomycin (n= 18). **B,** HyPer response to the 3 different stimuli. **C,** An Overlay of both signals, HyPer and the Ca^{2+} indicator. Arrows indicate time point of additions.

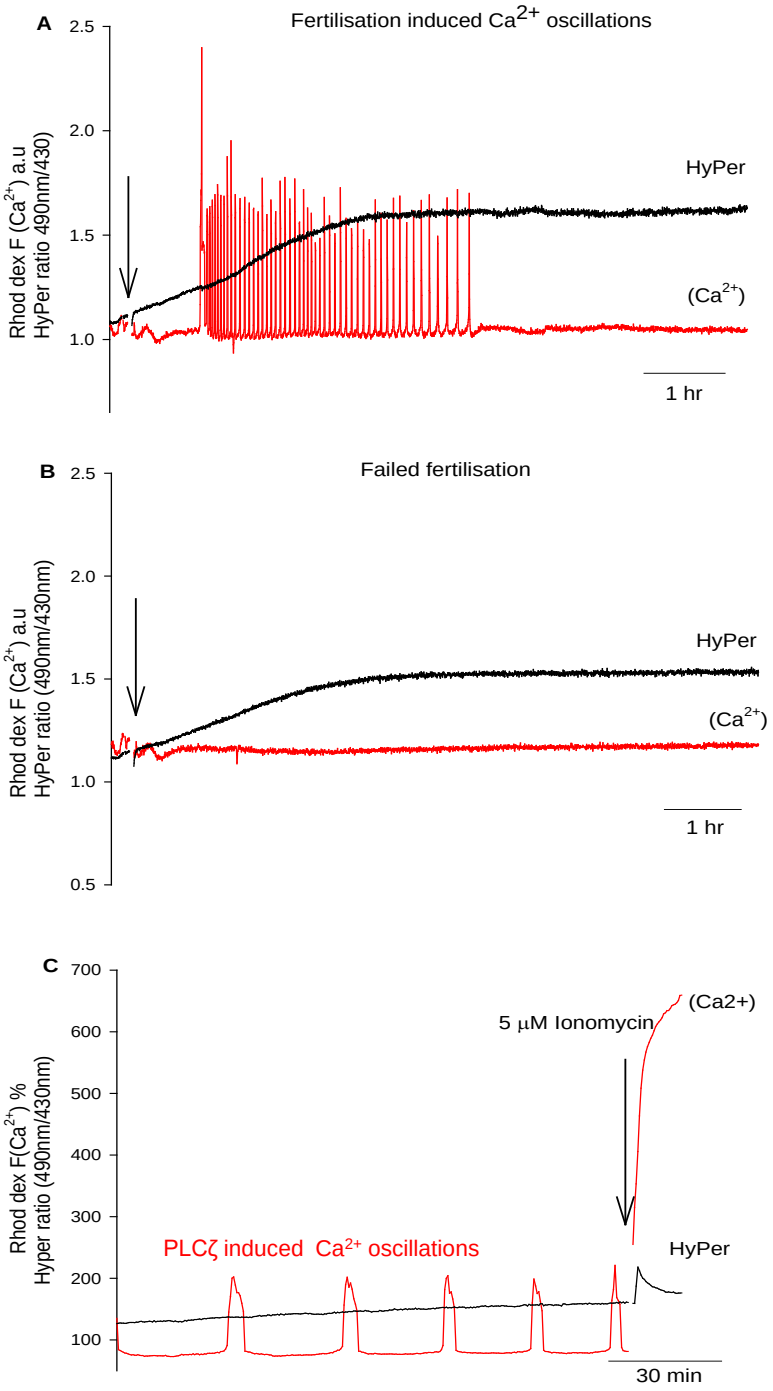

Figure 5.7 Ca^{2+} oscillations triggered by fertilisation and PLCζ and their effect on HyPer probe signal.

Changes detected in fluorescence signal and ratio for the Hyper probe and the Ca^{2+} indicator following, signal for both probes overlaid. A, In vitro fertilistaion (n= 22). B, failed fertilization (n= 12). C, Ca^{2+} oscillations induced via a micro-injection of 0.01mg/ml of hPLCζ followed by an addition of 5μM ionomycin (n= 27).

5.3.4 Germinal vesicle breakdown and egg maturation, does it cause any change in the ROS levels?

The role of the ROS in the course of in-vitro maturation of mouse eggs is still a matter of controversy (Morado et al., 2009). It was found that H_2O_2 can trigger GVBD in immature eggs, however, later it inhibited the ability of the egg to release the first polar body and initiated degeneration (Chaube et al., 2005). Here I decided to investigate if there were any fluctuations in H_2O_2 production during the course of mouse egg maturation. We were interested in examining oocytes undergoing germinal vesicle breakdown (GVBD) for their potential association with a possible transient increase in H_2O_2. Eggs at the germinal vesicle stage were obtained by dissecting ovaries in a media containing 100µM of IBMX then picking up the oocytes that have a well-defined GV. To assay for this possible natural ROS transient, oocytes at this particular developmental stage were subject to a high pressure injection system to inject the HyPer probe. The injection step was done in media containing IBMX to prevent premature GVBD. Subsequently, they were placed on the imaging system where IBMX was omitted from the imaging media. The temperature was maintained at around 37^{o}C +/- 0.5^{o}C via a heated chamber.

The fluorescence signal generated from Hyper was recorded by the system described elsewhere, the developmental progress was assayed by differential interference contrast (DIC) microscopy. The system allows for switch between the two modes of imaging and with the use of an approximate timeline, it can correlate changes in the fluorescence signal to developmental progress. A positive control was used for this experiment where the media for imaging had IBMX to prevent GVBD. The signal of both experiments with and without IBMX is shown in (Fig.5.8 A, B). There was a slight increase in the Hyper probe signal in oocyte in these experiments but this increase was not consistent and occurred regardless of whether oocytes underwent GVBD or not. The data suggests that there was no change in the level of ROS during the process of GVBD (maturation). We further examined this process by the use of the HyPer probe pre-incubated with DTT, and again the results were the same suggesting that no alteration of the ROS was evident (Fig.5. 8c).

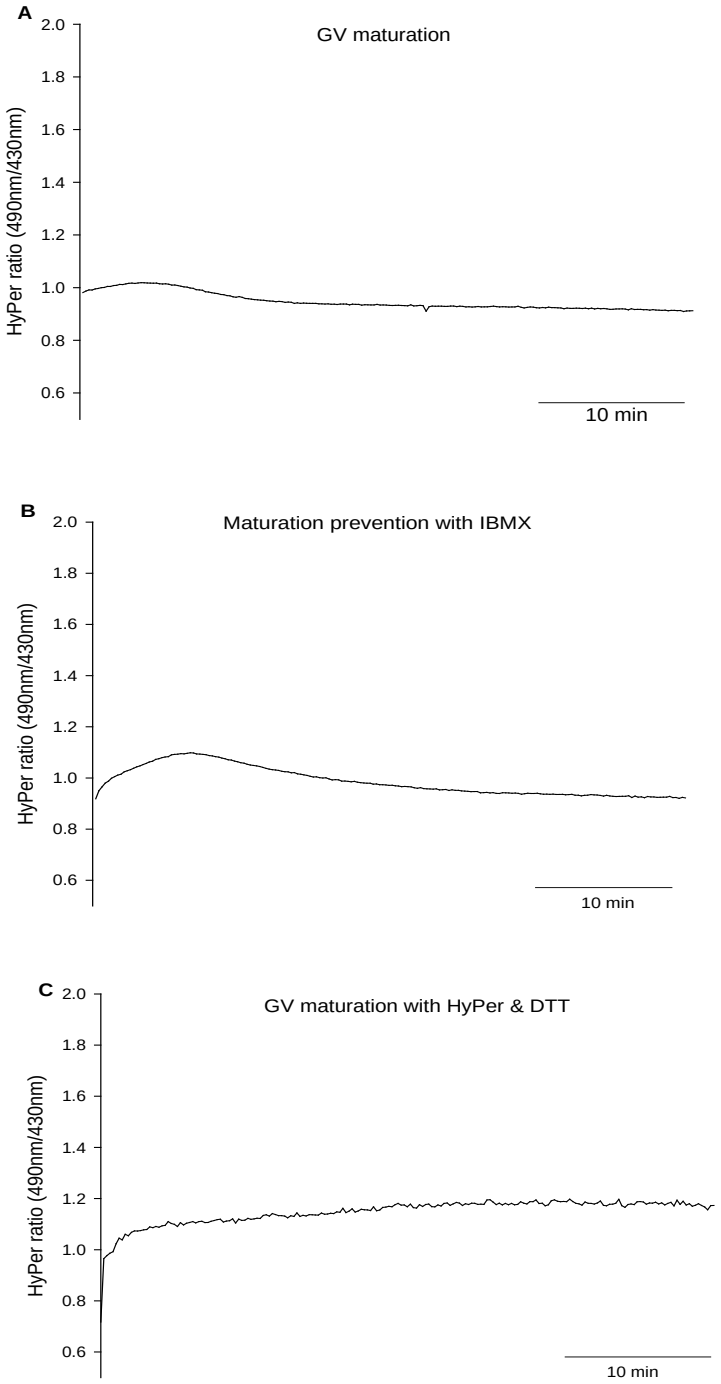

Figure 5.8 Germinal vesicle breakdown and prevention, effects on Hyper probe signal.

GVs were micro-injected with the HyPer probe. **A,** allowed to undergo GVBD (n= 20) and the signal compared to that of **(B)** GVBD prevention (n= 23). **C,** Fluorescence ratio of HyPer probe with added DTT detected in GVs during maturation (n= 19).

5.3.5 The contribution of mitochondria to the change of HyPer probe signal.

Mitochondrial un-couplers such as carbonilcyanide *p*-triflouromethoxyphenylhydrazone (FCCP), have long been used as a tool for investigating the role of mitochondria in cellular functions. As mitochondria plays a significant role in Ca^{2+} buffering capacity of the cell, mitochondrial inhibitors can be used in research of the cytosolic Ca^{2+} homeostasis. Mitochondria can either promote uptake or release of Ca^{2+} depending on their energy level. Mitochondria could also be a significant source of H_2O_2 (Murphy, 2009) and the production of ROS depends upon mitochondrial potential. Hence, reducing the mitochondrial potential with FCCP could reduce the production of H_2O_2.

This experiment was set up to investigate the source of H_2O_2 when external additions were omitted. HyPer probe was then introduced by high pressure injection. Eggs loaded with both indicators were placed on a preheated glass bottom dish after stripping the zona's off for egg stabilisation. A baseline for both signals was established for some time, in the presence of 1.7mM external Ca^{2+}, then 1µM of FCCP was added to the surrounding media. This addition caused a transient rise of the cytosolic levels of Ca^{2+} (Fig.5. 9), which was presumably was due to the depolarisation effect of FCCP on mitochondria and release of Ca^{2+}. The addition of FCCP was done in two steps, the first addition then followed by another after allowing for a recovery of the signal close to the baseline. In conjunction with the increase in cytosolic Ca^{2+} shown by the rise of the designated trace, we can identify a similar increase in the HyPer probe signal in terms of timing of the change. Indicating that the parallel change in HyPer probe signal is probably caused by a factor related to Ca^{2+} change which may be H_2O_2. However, this signal is not what is expected if mitochondria were producing significant resting H_2O_2.

We have used 10µM dipheneylene-iodonium (DPI) as an inhibitor of NADPH-oxidase inhibitor, which is another potential significant ROS producer in cells. DPI is non-specific in inhibiting NADPH oxidase-mediated ROS formation, as it inhibits other flavo-enzymes such as xanthine oxidase (XOD). Despite the unspecific mode of action, it was not effective in our experiments because it was unable to abolish the intracellular production of ROS. Following a parthenogenetic activation by 5µM ionomycin the same effect on the HyPer signal was detected in the presence of DPI.

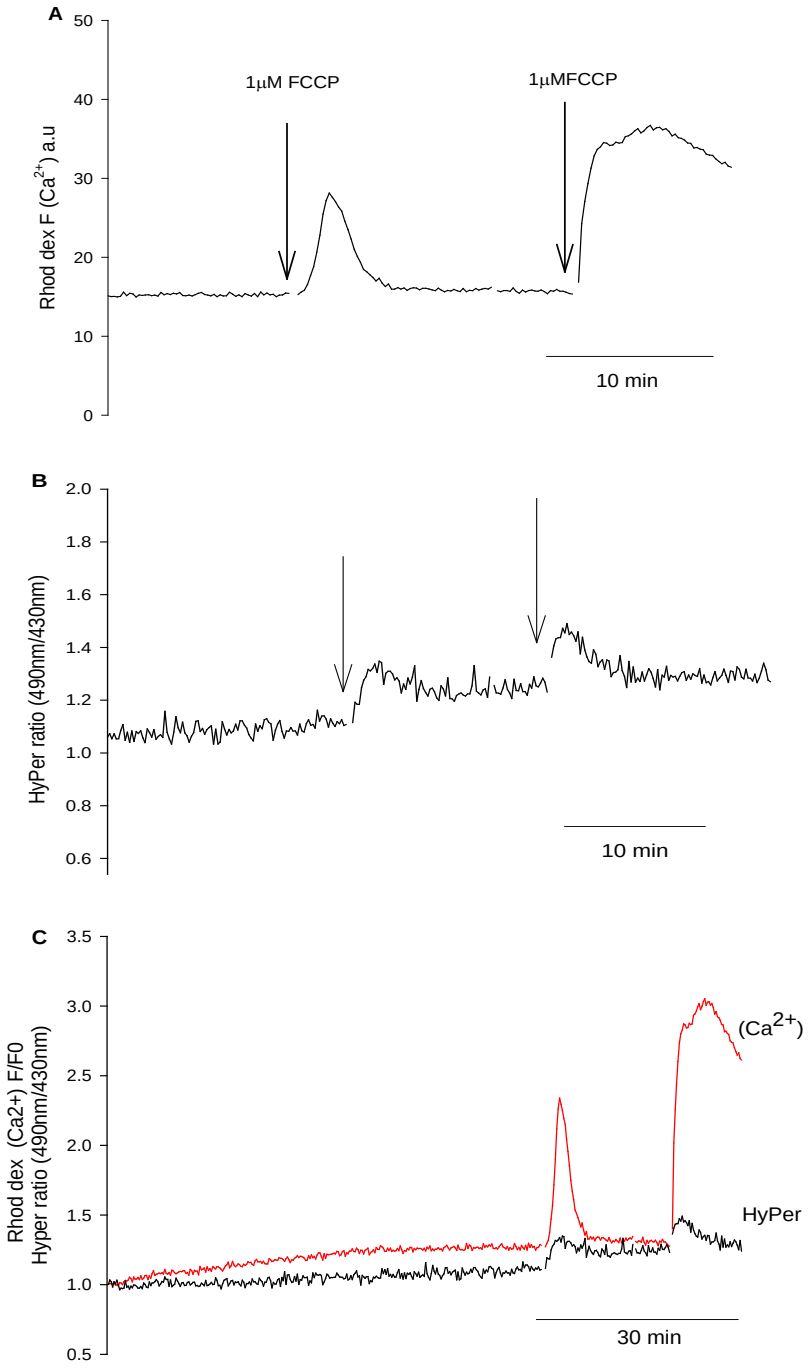

Figure 5.9 Mitochondrial contribution to changes detected in the HyPer probe signal.

Signal tracking for HyPer probe and Ca^{2+} during two consecutive additions of 1µM of FCCP. The second addition was done after the return of the Ca^{2+} signal indicator to baseline**(A)** arrows indicate to time of addition. **B,** Hyper fluorescence signal change after the two addition of FCCP. **C,** The overlay of both signals (n= 28).

5.3.6 Investigating the influence of the antioxidant system on HyPer signal.

5.3.6.1 Catalase inhibitor effect on the signal generated by HyPer

When compared to other ROS, H_2O_2 is considered to be the most stable and most significant in terms of concentrations. Characteristically it is uncharged and can freely diffuse between and within cells. Catalase is an essential enzyme that is found in all mammalian cells exposed to O_2. The enzyme catalyses the decomposition of H_2O_2 into water and oxygen. As an enzyme it has one of the highest turnover rates (Chelikani et al., 2004).

3AMT (aminotriazole) is a catalase inhibitor that has the ability to significantly abolish catalase's activity. In other studies it has been found that the highest level of the enzyme inhibition was obtained when a concentration of 50mM AMT was used. Therefore, when eggs were incubated with 50mM AMT this should only indirectly affect the levels of H_2O_2 by suppressing the activity of catalase. MII eggs were stripped from their zona's and placed in a preheated dish at (36.5-37.5^{o}C) containing 50mM AMT and incubated for about 1hr. After establishing a steady baseline, an addition of H_2O_2 was done. From (Fig.5.10 A, B) it can be seen that the after the addition of two different concentration of H_2O_2 (100µM H_2O_2, 1mM H_2O_2) the signal detected is almost identical to previous fluorescence signals when no AMT was used. This result has at least two interpretations. One is that catalase plays little role in the recovery of mouse eggs from a pulse of H_2O_2. The other is that the fluorescent signal change of HyPer is effected by other factor than the concentration of H_2O_2.

5.3.6.2 Inhibiting Glutathione peroxidise, does it have any influence on the HyPer signal generated?

We further examined the relationship between ROS eliminating machinery and the signal originating from HyPer probe. It was aimed to investigate the contribution of the antioxidant enzyme glutathione peroxidase (GPx) to the signal change produced (Shiba and Shimamoto, 1999). MII eggs were stripped from their zona pellucida using acid tyrode's and placed in a preheated dish containing HKSOM with a final concentration of 100µM of mercaptosuccinate which is a recognized inhibitor of GPx. After a designated time (1 hour) which is to allow for the uptake of the drug, an addition of H_2O_2 was made to the dish. Two concentrations of H_2O_2 were

separately tested (100 μM and 1M) figure 5.10(C, D). In each case there was a large rise in the HyPer ratio signal followed by a steady decline and he fluorescence imaging was terminated before the signal came back to baseline. The observed fluorescent signal resulting from the HyPer probe was again undistinguishable from mouse eggs that were untreated. These data and the above are surprising since both inhibitors of GPx and catalase failed to affect the HyPer signal changes induced by H_2O_2, It is possible that the signal change was not entirely due to an increase and decrease in H_2O_2 alone.

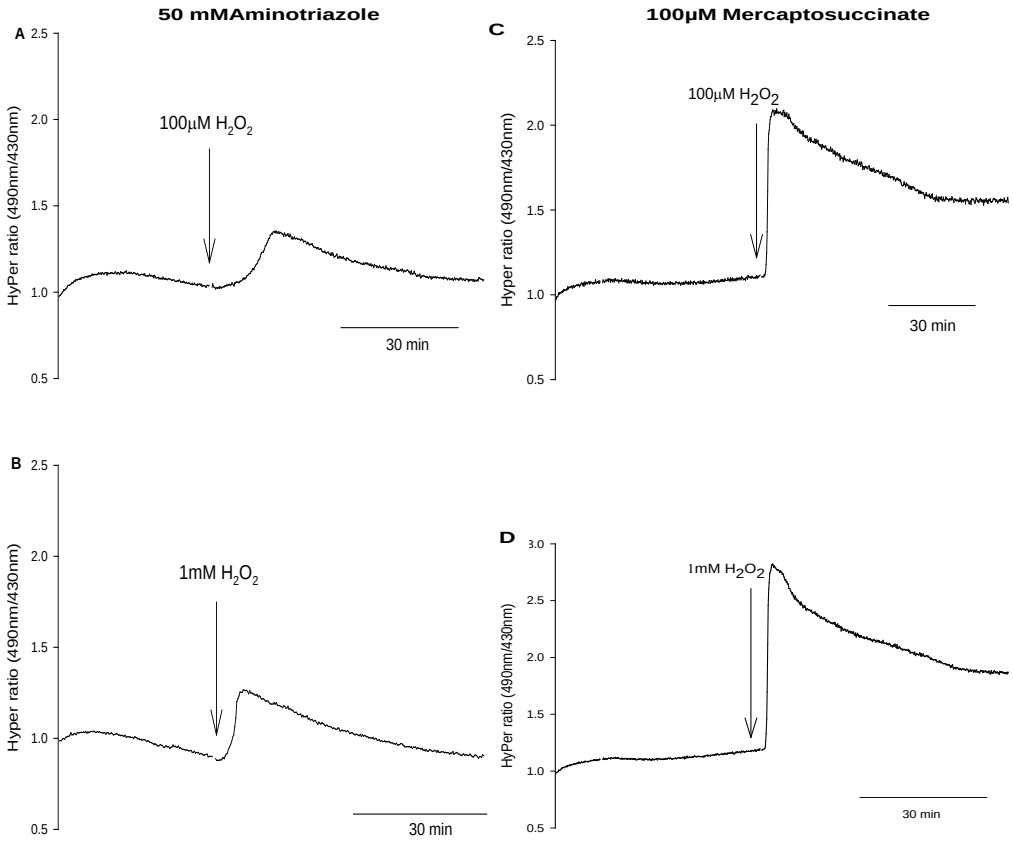

Figure 5.10 The influence of the antioxidant system on the HyPer probe signal.

Traces representing the use of 50mM AMT attempting to block the pH change detected by the HyPer probe (A, n= 19, B, n= 19). 100μM mercatosuccinate was used to investigate the cause of the pH detected by the HyPer probe (C, n= 17) (D, n= 21). Both experiments tested the effect of 100μM and 1mM of H_2O_2.

5.3.7 Intracellular pH during egg activation, does it influence the resulting HyPer signal?

A number of reports indicate that Ca^{2+} changes have the ability to cause an increase in the intracellular pH in certain cell types including mouse eggs (Kline and Zagray, 1995). In the light of reports indicating a possible pH sensitivity of the HyPer probe, I decided to examine the possibility of a contribution of cytosolic pH to the signal change of the HyPer probe in some of the types of experiments reported above. Eggs were micro-injected in M2 media with 5µM of the dye SNARF that was covalently bonded to 10.000MW dextran. This pH indicator was shown to remain within the cytoplasm of unfertilized mouse eggs by House 1994 (House, 1994). Following the high pressure injection, eggs were allowed to recover for a short period of time. Each experiment was conducted by a random pick of about 9-12 eggs at a time. Zona's were stripped and eggs placed in a temperature controlled glass bottom dish approximately at 37^{o}C. Using the fluorescence measuring system described elsewhere, one excitation wavelength of 580nm and emission wavelengths of >620nm and at 580nm were recorded for SNARF. The ratio of the emission intensity was calculated by dividing both images subsequent to a background subtraction. The change in the emission intensity was mainly pH dependant.

5.3.7.1 Does the presence of a pH indicator affect the performance of the HyPer probe?

In order to determine whether both indicators would have any interaction or affect the performance of one another, both have been micro-injected separately. First the pH indicator was micro-injected and after a brief period of recovery the eggs were micro-injected with the HyPer probe. Eggs were washed through several drops of M2 then allowed for a second round of recovery. Once micro-injected and recovered, eggs were transferred to the preheated dish and a baseline was observed. Once the baseline for both indicators was established an addition of 10mM NH_4CL was done and once the signal response had peaked it was washed out (Figure 5.11). Additions of NH_4Cl are commonly used to raise intracellular pH in cells. The system in use allows for multichannel switch, as both indicators are ratio-metric necessitating a four channel wavelength emission measurement. Figure 5. 12 (A,) shows the response of both indicators to the induced alkalosis as they respond by a sharp increase in the signal generated. When both are signals overlaid they

demonstrate a synchronized response to NH4CL, suggesting that the HyPer probe is influenced by the change in the intracellular cytosolic pH.

5.3.8 Parthenogentic activation, does it cause a shift in the egg pH?

Now that it has been established that a rapid pH change in mouse eggs can possibly influence the HyPer probe signal, it was important to find out if the increase in cytosolic Ca^{2+} influences the pH. To measure the changes in egg pH during parthenogenetic activation using 5µM ionomycin, eggs were micro-injected with both indicators as previously described. Once a baseline signal was established for both indicators (SNARF-dextran & HyPer) an addition of ionomycin was done so that the final concentration to the surrounding media would be of 5µM. It can be seen from Figure 5.12 (B) that there was a rapid change response triggered by the ionmycin addition and a synchronized signal response was seen with both signals (SNARF-dextran & HyPer) recorded as demonstrated. The signal change in both probes was of a quick increase that once it reached a peak it declined rapidly. However, the signal remained above the baseline that was detected before the ionophore addition. From this result it is evident that ionomycin causes a pH change and that the HyPer probe is highly influenced by such induced pH changes.

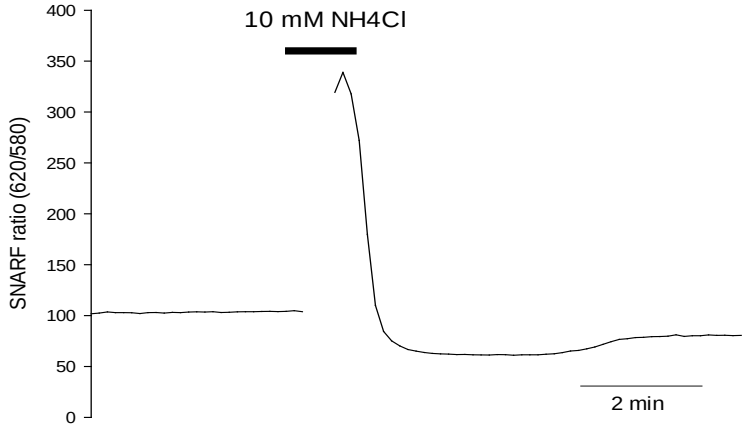

Figure 5.11 SNARF dye response to addition of NH4Cl in mouse eggs.

The response of SNARF following micro-injection into mouse eggs and the addition of 10mMNH4Cl, bar indicates the addition and the washout NH4Cl (n=23).

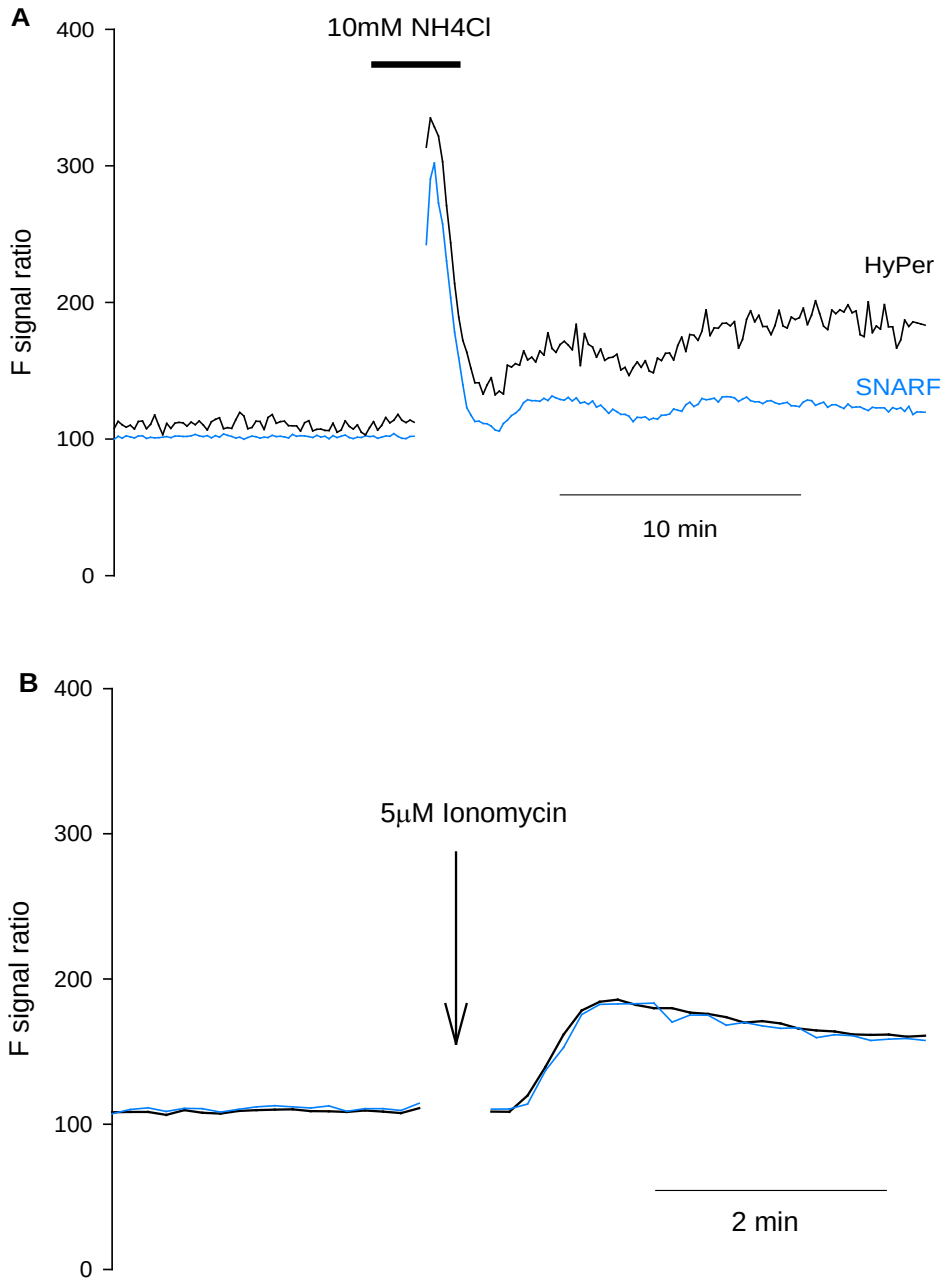

Figure 5.12 SNARF and HyPer signal detection in mouse eggs.

A, An overlay of fluorescent signals for HyPer& SNARF in response to addition and washout of 10mMNH4Cl (n= 17). **B,** HyPer (black trace) and SNARF (blue trace) fluorescent signal ratios in response to the addition of 5mMionomycin (n= 26).

5.3.9 Investigating the pH change during Ca^{2+} influx throughout parthenogenetic activation of mouse eggs.

Mouse eggs (MII oocytes) gain their ability to regulate their intracellular pH right towards the end of their growth (Erdogan et al., 2005). Two exchangers are accountable mainly for this control, the HCO^-_3/CL^- exchanger which by its action can increase or decrease the intracellular pH, and the Na^+/H^+ exchanger which works to increase the intracellular pH. The upcoming sets of experiments were set to determine the basis of the pH change detected by SNARF, which has influenced the probe under investigation (HyPer).

5.3.9.1 Activation of mouse eggs in a media containing 2.5mM Amiloride.

In order to search for the cause of the pH shift I investigated the most common reasons behind a cellular pH change. The sodium-dependant channels or to be more specific a Na^+/H^+ antiporter was the first studied. This antiporter has a high affinity for the diuretic amiloride. Amiloride has the ability to irreversibly prevent the movement of Na^+ through Na^+/H^+ exchange. Eggs were treated with 5µM ionomycin after a baseline acquisition, with the media containing 2.5mM amiloride. Both fluorescent signals had an increase in their intensity. With the SNARF signal increase outlasting that of HyPer probe. However, both signals declined and met at a point above the initial baseline for both probes. The shift in the signal after the ionophore addition indicated that there was a shift in the pH which affected the HyPer probe signal in parallel as seen in figure 5, 13A. This result indicates that this exchanger was not the cause of the change in pH detected in our experiments.

5.3.9.2 Activation of mouse eggs in a media containing 50µM DIDS

The next set of experiments was designed to examine whether the pH change observed during the parthenogenetic activation protocol using 5µM ionomycin was bicarbonate dependant. More specifically, is the change caused by a sodium-dependant or independent HCO_3^-/Cl^- exchanger. To do this we used the drug DIDS which is a recognized inhibitor of this exchanger and is effective in mouse eggs (Baltz et al., 1991).

After obtaining a baseline signal for a brief period of time in a medium containing 50µM of DIDS, a treatment with 5µM Ionomycin was done. Again here there was a rise in both fluorescent signals, but the change for the SNARF signal was bigger than that detected for the HyPer probe. Both signals did not return to the pre-

ionomycin baseline signal. The treatment resulted in an increase of the intracellular pH (Fig.5.13B). As the pH change was not prevented by the use of DIDS, it excludes HCO_3^-/Cl^- exchanger as the cause behind the change.

5.3.9.3 Activation of mouse eggs during the inhibition of H^+ ATPases.

Until this point examining the common pH regulation mechanisms could not explain the shift in the pH detected and measured by the SNARF probe during the treatment with 5µM ionomycin. So next, my attention was turned to the vacuolar-type ATPases. They are considered the main candidate for generating acidity in the central vacuolar system. Bafilomycin, a macrolide antibiotic (Werner and Hagenmaier, 1984) is used specifically to inhibit H^+ ATPases in a nanomolar concentration. After establishing a baseline for the signal in a media containing 500nM Bafilomycin a final 5µM ionomycin was attained. Figure 5.13(C) demonstrates the response recorded and shows that both probes responded by a sharp increase in their fluorescence signal that started to decline slowly after it reached a peak. The fluorescent signal increase in this case was almost the same for both probes. These data suggest that bafilomycin could not block the changes in both signals, for the pH probe and ROS probe once again.

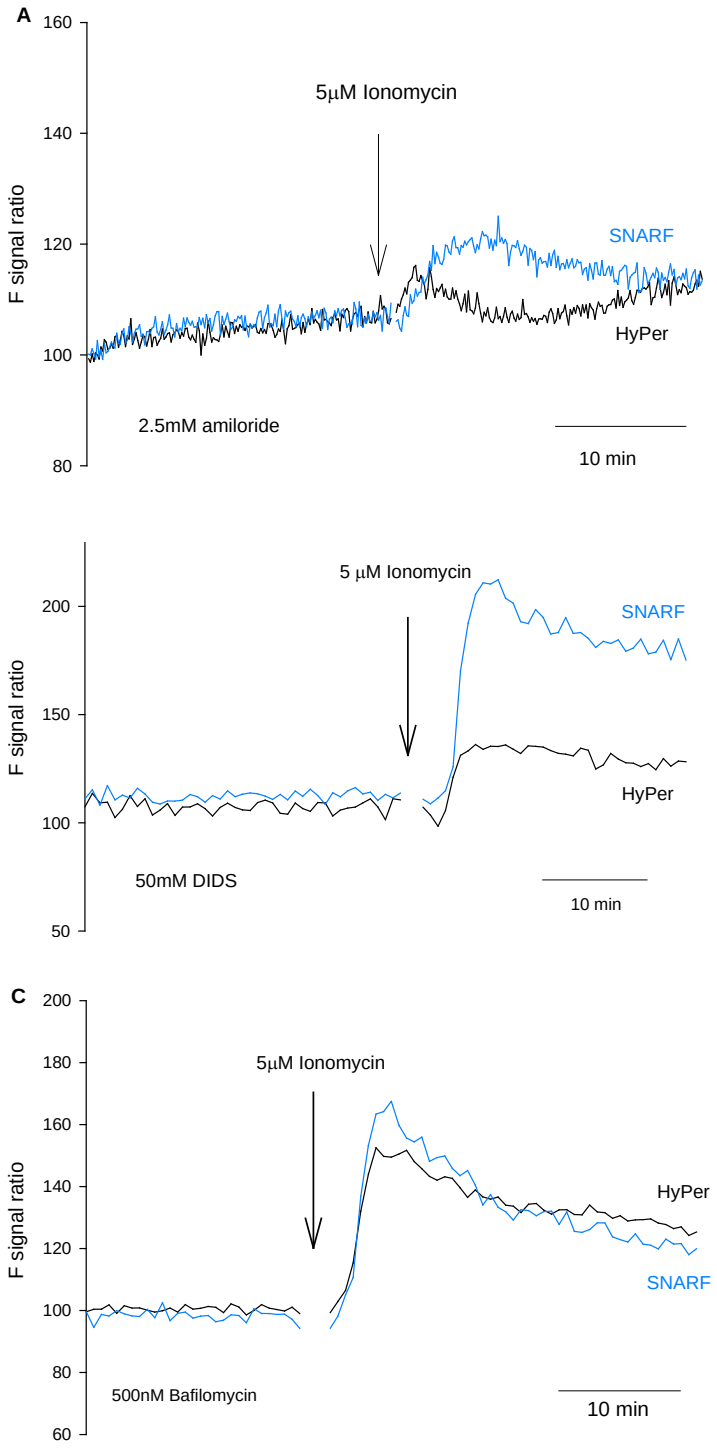

Figure 5.13 Traces representing the investigation into the cause of pH changes in mouse eggs detected during artificial activation by ionomycin.

Mouse eggs are micro-injected with both indicators HyPer & SNARF. **A, B, C** are fluorescent signal ratios overlaid for both probes in response to the addition of 5μM ionomycin in the presence of 2.5mM amiloride, 50mM DIDS and 500nM bafilomycin respectively. Arrow indicates addition of 5μM ionomycn (n= 22, 27, 19) for A, B, and C respectively.

5.4 Discussion

Oxygen, an essential molecule for aerobic life, is essential in many ways. It serves as a terminal acceptor of electrons in the very vital process of respiratory chain reaction, as well as a regulator to many intracellular and extracellular signalling pathways. Reactive oxygen species (ROS) are produced in a tightly controlled manner. However, in circumstances where the balance between production and elimination leads to an excess of ROS, it gives rise to pathological conditions. Oxidative stress is a term used to identify the non-specific damage that occurs to proteins, lipids and DNA due to excess ROS. In field of reproductive cell biology, it is well documented that Ca^{2+} oscillations during fertilisation induce an increased mitochondrial activity in terms of an increase in ATP production (Campbell and Swann, 2006). A key source for ROS in the cell is the mitochondria (Turrens, 2003) and the mouse egg is of no exception. H_2O_2, a member of the ROS has been shown to mediate programmed cell death (PCD) in the blastocyst stage of embryos (Pierce et al., 1991). Likewise, human embryos that had high levels of H_2O_2 displayed sign of PCD (Jurisicova et al., 1996). The aforementioned was the reason behind the interest in studying the effect of Ca^{2+} oscillations, and transients induced by different means, upon the production of H_2O_2 and thereafter upon embryo development. There are studies suggesting an increase in levels of H_2O_2 as embryos progress to the 8-cell stage after fertilisation or after parthenogenetic activation (Nasr-Esfahani et al., 1990a). However, most of these studies used the fluorescent probe 2', 7'-dichlorodihydrofluorescein (DCHF), which fluoresces after oxidation by H_2O_2 which yields 2', 7'-dichlorofluorescein (DCF). It is thought that the level of DCF is produced in linearity with the level of H_2O_2 present within the eggs (Nasr-Esfahani et al., 1990b). On the other hand, these reported compounds have two major drawbacks that make any claims resulting from their use questionable. The first drawback is that the dihydro-compounds in themselves are highly photosensitive to variable degrees. Therefore in the presence of excitation light they tend to autooxidise to produce significant background fluorescence in the absence of any detectable ROS (Afzal et al., 2003). For that reason when these compounds are used for ROS detection, the increase in fluorescence intensity detected is a sum of auto-oxidation of the probe along with the presence of ROS. Another credible argumentative point against the use of this compound is the lack of specificity for ROS; generally they are thought as to be indicative of the overall state of the oxidative species (Hempel et al., 1999). These probes originating from DCFH compounds are widely used as a

ROS probe despite the concerns about what fact it is really measuring. Therefore, the demand for novel probes that have the ability for detecting individual ROS with high levels of specificity and sensitivity is increasing.

Belousov et al in 2006 reported the use of a novel H_2O_2 fluorescent probe that has the ability to detect intracellular levels of H_2O_2. The basis of the probe was built around the regulatory domain of *Echerishia coli* OxyR which is specific for H_2O_2. This genetically encoded biosensor consists of circularly permuted yellow fluorescent protein (cpYFR) inserted into the OxyR domain. The conformational changes that occur to the probe during exposure to H_2O_2 are the origin behind the fluorescent signal change (Belousov et al., 2006). The HyPer probe has been reported to be sensitive to sub-micromolar levels of H_2O_2 with the advantage of being a ratio-metric sensor, so the signal change would be independent of the level of probe injected.

I have investigated the first use of this new H_2O_2 specific sensor in any species of egg. My experiments initially demonstrated that the probe is able to record changes in its fluorescence in a response to a range of H_2O_2 concentrations (100µM, 1mM and 10mM). The authors of the original paper claim that the probe was able to sense a level of 25nM for the isolated probe while the sensitivity was reduced to 5-25µM in *E.coli*. Next, we went on to test if the probe would be able to sense any changes in H_2O_2 levels in the eggs during parthenogenetic activation. That was done via the use of 5µM Ionomycin which induces relatively a large single cytosolic Ca^{2+} transient. To correlate cytosolic Ca^{2+} changes to H_2O_2 levels, injection of two probes into the eggs was done. Measuring the changes in the fluorescence level of both probes revealed that at the point where the Ca^{2+} indicator starts to show an increase, the HyPer probe also demonstrates an increase in its fluorescence. However, when we followed this pattern of change to see if it extends to other causes of Ca^{2+} changes in mouse eggs the picture was not so clear. In-vivo fertilisation could not induce any changes in HyPer probe signal despite the fertilisation induced Ca^{2+} oscillations being observed quite distinctly with the rhod-dextran probe. Notably the fluorescence of the HyPer probe in eggs that were fertilised and those that did not fertilise showed no marked differences.

An immature oocyte is also thought to be significantly affected by during *In-vitro* maturation (IVM) by oxidative stress. Four transcripts encoding the main enzymatic antioxidants (GPx, Cu/Zn-SOD, Mn-SOD and G-glutamylcysteine synthetase) have

been detected not only in mature mouse eggs but in immature eggs as well (Mouatassim et al., 1999). This implies that this defence system is important for the IVM process. For these reasons I examined the IVM process, from germinal vesicle stage to an MII egg. The eggs were imaged via DIC during the breakdown of the GV while recording the HyPer probe fluorescence signal. Again the results failed to show any significant change that could indicate a detectable increase in the production of H_2O_2 during IVM. The controls that these were measured against were immature oocytes with intact GVs that were imaged in IBMX to halt the maturation process. To my knowledge there are no reports on changing ROS levels during GV breakdown

Next we investigated the role of mitochondrial contribution to the change in the signal of HyPer probe. It was earlier thought that following fertilisation induced Ca^{2+} transients there would be an increase in the pH. This phenomenon was considered to be a universal act across species (Steinhardt et al., 1974). It has been reported that in bovine, porcine and murine eggs during their activation using ionophores namely A23187 and 7% ethanol they all showed a change in their cytosolic pH. The exact mechanism of the pH change was further investigated but the cause is yet to be identified (Ruddock et al., 2000). However, Kline and Zagray in 1994 reported contradictory results in mouse eggs (Kline and Zagray, 1995). Furthermore, it has been established that after GV breakdown, unfertilised eggs and in-vitro fertilised eggs had no difference in their baseline pH levels (Phillips and Baltz, 1996).

The results presented failed to demonstrate a persistent response to the increase in H_2O_2, while Ca^{2+} transients triggered by ionomycin led to an increase in HyPer probe fluorescence, *in vitro* fertilisation failed to do so. We went on to investigate the effect of inhibiting the catalase activity by 3AMT. 3AMT has the ability to immediately inhibit the enzyme at a rather high concentration which can be reversible (Yoshioka et al., 2006). After a suitable dilution, it was found that the signal generated by HyPer probe after the addition of H_2O_2 was similar to the change seen when no catalase inhibitor was used. The lack of any detectable difference in the signal indicated that the change was possibly not due to changing levels of H_2O_2. Furthermore, no detectable difference in the signal was seen when a glutathione peroxidase inhibitor was used. We investigated the role of mitochondria on this signal via the application of a mitochondrial un-coupler (To et al., 2010). FCCP application led to a Ca^{2+} increase due to the depolarisation effect on

mitochondria. This signal generated from the HyPer probe matched that of the Ca^{2+} transient effect on the Hyper signal using Ca^{2+} ionophores.

It has been reported the Hyper probe is pH sensitive (Belousov et al., 2006). This sensitivity is related to the cpYFP integrated into the probe, since cpYFP is a fluorescent protein that is pH sensitive (Wang et al., 2008, Bilan et al., 2013). Therefore, given the probe's pH sensitivity it is plausible that at least part of the signal generated in eggs is due to a change in the pH. To validate this hypothesis, further experiments were carried out. Observing the signal generated with the HyPer probe along with a pH sensitive probe were thought to be informative. HyPer and SNARF-dextran are both ratio-metric probes with molecular weights of 52 and 70.000 kDa respectively. The concomitant use of both probes in mouse eggs was done to enable the measurement of the HyPer probe signal versus any change in the pH probe. To my knowledge, the results reported here are the first with the use of these probes together in any egg. The data generated suggests that they are detecting the same event which is a change in the pH level rather than a change in the level of ROS. This is in agreement with the critical evaluation of a similar mitochondria HyPer probe (mt-cpYFP). Schwarzlander et al in 2011 showed that the superoxide flashes detected by this probe were altered by manipulating the mitochondria pH. With our set of experiments the questions remain: are both probes reporting the same event? More specifically are they reporting changes in pH levels or in H_2O_2 levels? Having said that, it has been documented previously that there is no pH change during in-vitro fertilisation (Kline and Zagray, 1995). Similarliy, no pH change was detected during parthenogenetic activation by 7% ethanol (Phillips and Baltz, 1996). On the contrary, studies in pig have revealed an increase in intracellular pH during parthenogenetic activation using 7% ethanol and ionophore A23187 (Ruddock et al., 2000). Our results have revealed that there is a measurable pH change during parthenogenetic activation using 5micromolar ionomycin, which comes in disagreement with both previous reports on mouse eggs. Before proceeding to any conclusions, a short series of experiments were performed in an attempt to explain the pH change detected. Firstly, the common pH regulatory mechanisms were examined. Inhibiting both the amiloride-sensitive Na/H antiport and the HCO3/Cl exchanger demonstrated no change in the signal recoded. Previous reports suggested that Ca^{2+} ionophores have the ability activate vacuolar-type H+ ATPases (Ruddock et al 2000). For that, we extended the experiments to examine a less common regulatory mechanism. If Ca^{2+} release or influx induced by

ionomycin turns on the activity of H^+ ATPases in our mouse eggs, by using Bafilomycin A1 it should be possible to block the pH change detected without affecting the Ca^{2+} influx. In contrast to this prediction, upon the addition of 5µM ionomycin, bafilomycin prevented the return of both signals to baseline and did not prevent the change. Further experiments could have been conducted to examine the possibility of whether it is Ca^{2+} increase within the cell, or was it the activity of H^+ ATPases behind the last result. However, we felt that pursuing this matter further would lead to a deviation from our primary interests.

In conclusion, our results have indicated that during parthenogenetic activation of mouse eggs via ionomycin there is an increase in intracellular pH. It is clear that we have not managed to find the cause of this change nor we could prevent it. Ca^{2+} ionophore activation leads to a pH change that was independent of external sodium or bicarbonate but otherwise due to an initial rise in of cytosolic Ca^{2+} levels. It would be interesting to further study which mechanisms are behind the pH change that I detected. Unfortunately, our primary point of interest was studying the effect of Ca^{2+} changes during fertilisation and parthenogentic activation upon the levels of ROS and specifically H_2O_2. The data in this chapter suggest that Hyper is not usable for this purpose. Regrettably, until other probes are made available this project could not be taken any further.

Chapter 6

GENERAL DISCUSSION

6 General Discussion

6.1 Overview

The thesis that I have presented, is a continuation into the research being carried out on sperm PLCζ. Several findings have resulted from my work. I have shown the efficiency of hPLCζ protein in causing Ca^{2+} oscillations in mouse eggs that have failed to activate following an ICSI procedure. Currently there is no method to identify a sperm's level of the activation factor prior to injection. So, I have also presented results suggesting that the protein can be used in all cases of ICSI, without any deleterious effects on subsequent embryo development. However, these results were from experiments conducted on mouse eggs, further evidence will be needed from examining the response of human eggs. The effect of the vital tag (Nus-A) for the protein was looked at as well, no detrimental effects on pre-implantation development of mouse embryos was found.

In this chapter I will be discussing current parthenogenetic protocols used to cause Ca^{2+} oscillations in mammalian eggs, including mouse and human eggs. Similarly, I will include a comparison of various activation protocols against hPLCζ protein. I will include as well a discussion for the challenges that could face the introduction of hPLCζ protein into clinical practice. A separate section had an aim of developing a reproducible method for injecting the hPLCζ protein during ICSI. In this section I have showed preliminary results indicating that a single co-injection technique is likely to be successful. However, a substitute for the current PVP molecule used to increase the medium viscosity is required. An increase in medium viscosity is required to reduce sperm motility without the formation of aggregates that could affect the functionality of the protein. A number of experiments were also included in this thesis attempting to explain how Ca^{2+} oscillations would have an extended effect on embryo development subsequent to their cessation. The experiments were focusing on the possibility of the effect being delivered through changes of ROS levels. However, the probe (HyPer) used to investigate such a cause and effect has proved to be unreliable because as it proved to be very sensitive to pH changes. Therefore, no conclusions could be made until further probes are available.

6.2 Modern IVF and Egg Activation Problems

The success of fertilised mammalian eggs to further develop is mainly dependant on orchestrated operation of genetic and developmental programmes. Whether an embryo would be able to advance to the pre-implantation stage or arrest at early stages, is highly dependent on factors present at or before the 1-cell stage (Hardy et al., 2001). However, prior to fertilisation other factors could affect success rates such as the presence of chromosomal abnormalities (Munne et al., 1995), egg quality in what is known as oocyte maturity (Moor et al., 1998), as well as other factors that could negatively impact the ability of an egg to fertilise. Since the early 1990's it has been shown that the sperm was not a prerequisite for egg activation (Swann, 1994). The challenges for developing artificial activation protocols were caused by an incomplete understanding of egg activation as a process. However, with time it has become clear that the common factor for all approaches was to induce a cytosolic Ca^{2+} increase/increases in the egg (Whitaker, 2006). Ca^{2+} was found to be influencing not only the immediate release of eggs from arrest, but extended to affecting the development potential of embryos in mouse and rabbit (Ozil, 1990, Ozil and Huneau, 2001, Bos-Mikich et al., 1997). If Ca^{2+} transients were manipulated during their application in terms of frequency and duration, an increase to a certain limit will cause an enhancement of pre-implantation embryos undergoing compaction (Ozil, 1990). Not only the number of embryos undergoing compaction would increase, but the quality of rabbit post-implantation embryos in terms of somite and heart formation could also improve (Ozil and Huneau, 2001).

Internal release and external influx of Ca^{2+} was successful in releasing the egg from its meiotic arrest. Ca^{2+} ionophores such as ionomycin, or 7% ethanol were amongst the agents that promoted a cytosolic Ca^{2+} increase (Cuthbertson and Cobbold, 1985). A divalent cation (Sr^{2+}), also showed to cause credible egg activation in mouse eggs (Kline and Kline, 1992). Moreover, this cation triggers the closest response to that of sperm during fertilisation. In fact, Sr^{2+} is the only known egg activator that triggers a series of Ca^{2+} oscillations during its application (Kono et al., 1996), while other agents can only induce a montonic single Ca^{2+} rise. The efficiency of the montonic Ca^{2+} rises in causing egg activation have been questioned, although they have been reported to cause egg activation in mammalian eggs including human eggs (Borges Jr et al., 2009b, Nasr-Esfahani et

al., 2010). In a direct comparison of various activating agents on mouse eggs, the results showed that Sr^{2+} has the highest efficiency in activating eggs and supporting further embryonic development when compared to other agents such ionomycin (Vanden Meerschaut et al., 2013b). My results have supported these previous studies since Sr^{2+} was superior to other artificial activators apart from PLCζ, in terms of that blastocysts development. The results emphasise the importance of the repetitive nature of the Ca^{2+} increase. However, Sr^{2+} treatment needed a supplementary action such as sperm introduction into eggs or cytochalsin treatment, otherwise it could not support early embryonic development (Bos-Mikich et al., 1997). That is explained by the need to have a diploid embryo. Not much success has been seen in the case of Sr^{2+} use in human eggs. A number of reports have shown that IVF and ICSI success rates can be enhanced with the use of Sr^{2+} (Yanagida et al., 2006, Kyono et al., 2008). However, studies using Sr^{2+} as an artificial activator failed to show Ca^{2+} transients as a result of its use in human eggs (Rogers et al., 2004, Heindryckx et al., 2008). In fact, whilst Sr^{2+} remains an effective activator and trigger of embryo development in rodent eggs, its effects have not been reproduced in eggs of any other non-rodent species including human. The cause of this species specific effect remains unknown.

The monotonic Ca^{2+} rise triggered by the use of Ca^{2+} ionophores can lead to successful early embryo development in sheep, pig, cattle and mouse. However, success rates are of moderate efficiency to say the least. It is unusual for an ionophore to be used as a sole egg activator as it is usually combined with a protein synthesis inhibitor such as puromycin. The aim is to ensure an egg release form the meiotic arrest which is maintained by the high levels of MPF. MPF in turn is kept at high levels by the turnover of cyclin B and that is where the action of protein synthesis inhibitors. Preventing the turnover of cyclin B reduces the level of MPF and therefore releasing the egg from its meiotic arrest. Freshly ovulated human eggs were activated via a single Ca^{2+} transient and went on to develop up to the blastocyst stage (Lin et al., 2003). However, this success reported, was only seen in one egg out of a total of six, and eggs were subject to an extended period of 15minutes to ionomycin. The ionophore treatment came subsequent to a sham ICSI procedure which would have caused an additional rise in Ca^{2+} (Lin et al., 2003). I have shown that in mouse eggs, embryos had the least ability to further develop when activated using ionomycin albeit the initial high activation rates to the pronuclear stage. The use of other parthenogenetic protocols in human eggs, such

as the use of protein synthesis inhibitors (Puromycin/cycloheximide) in conjunction to a monotonic rise of Ca^{2+} promoted early cleavage(De Sutter et al., 1992). Others reported that blastocysts were never obtained using this method of activation (Lin et al., 2003).

One of the embryo quality markers is the degree of fragmentation detected in the blastocysts. A level of <10% is considered as minor and can still result in embryos that have the ability to implant and continue into a term pregnancy. A fragmentation below 10% has results of implantation and continuation to term in rates similar to that of embryos that had no signs of fragmentation. However, any level beyond 10% would have a negative influence on implantation and documented clinical pregnancy rates (Giorgetti et al., 1995, Ziebe et al., 1997). Fragmentation is not the only predictive indicator for embryo quality, maternal age (Giorgetti et al., 1995) and the blastocyst's inner cell mass number (ICM) are other important factors(Richter et al., 2001). Moreover, the ICM number was an effective method in deciding on the most competent embryos that would improve pregnancy rates in IVF (Liu et al., 2014). Studies have shown that the method of parthenogenetic activation influences the degree of apoptosis in mouse embryos. In a comparison between Sr^{2+} (Ca^{2+} oscillatory change) and a protein synthesis inhibitor (cycloheximide, no Ca^{2+} change) there was a significant difference in their apoptosis degree in favour of Sr^{2+} (Rogers et al., 2004). This indicates again the importance of the oscillatory nature of the Ca^{2+} changes. When different levels of Ca^{2+} oscillatory activity were examined in their ability to support embryo development to the blastocyst stage, it was found that the number of Ca^{2+} spikes influenced the ability of embryos to reach the pre-implantation stage and not the activation rates (Yu et al., 2008). It has to be noted that other down-stream effects of the Ca^{2+} oscillatory pattern could not be excluded from influencing the capability of embryo development. In this regard my results are consistent with previous results. I have shown that the number of Ca^{2+} spikes had a significant difference between the embryos that arrested at the two cell stage and the eggs that further developed to the morula stage. Generally, it can be concluded that the oscillatory activity of Ca^{2+} during egg activation could be at least in part responsible for some of the later effects on development such as the level of fragmentation and ICM cell number detected in blastocysts defining embryo quality.

Since the introduction of IVF procedures (IVF, ICSI) there has been an ever increase in studies evaluating differences between both procedures in terms of Ca^{2+} oscillations, activation rates, gene expression pattern and clinical pregnancy rates (Pregl Breznik et al., 2013, Sato et al., 1999a). The search of difference was based on the sperm incorporation mechanism since the initial location of sperm is substantially different in IVF and ICSI (Sutovsky et al., 1996). In terms of gene expression there was no significant difference between both procedures (Bridges et al., 2011). Again both techniques displayed a first Ca^{2+} spike that was higher and lasted longer than the subsequent spikes, however, in ICSI the first spike outlasted the first spike generated in IVF. The difference in the duration of the first spike had no significant difference on the down-stream effects of Ca^{2+} changes (Ozil et al., 2005). The ICSI procedure unlike IVF includes a mechanical reversible damage to the egg membrane, which causes by itself a Ca^{2+} rise (Sato et al., 1999b, Tesarik and Sousa, 1994, Nakano et al., 1997), therefore the initial spike in ICSI could be a sum of two Ca^{2+} rises. In mice, it was found that the overall duration of Ca^{2+} oscillations was less in ICSI than those in IVF (Kurokawa et al., 2004). However, in a study by Sato MS et al on the effect of ICSI procedure on subsequent Ca^{2+} oscillations it was found that the main effect was on the first Ca^{2+} spike induced by the sperm. The relation was an inverse one, the larger the first Ca^{2+} rise due to the injection the smaller the first spike resulting from the release of the sperm factor. The rest of the Ca^{2+} pattern was unaffected by the procedure (Sato et al., 1999a). ICSI as a procedure had inconsistent results across mammalian species. It is established that equine embryos display a low frequency of Ca^{2+} oscillations for a short duration after ICSI, hence the very few embryos progress to the blastocyst stage (Bedford et al., 2004). Bovine eggs are another example of failure of the ICSI technique to produce embryos that are capable to develop to blastocysts. The reason behind such failure being the insufficient Ca^{2+} oscillations triggered after injecting the sperm (Malcuit et al., 2006). The failure is mostly thought to lie in the ‘compromised’ activation of the sperm factor within the egg after ICSI, leading to a defective pattern of Ca^{2+} oscillations and a premature termination of embryo development (Bedford et al., 2004, Malcuit et al., 2006). These data all suggest that there is still more to learn and more to improve on with the ICSI procedure.

6.3 PLCζ, a common trigger for egg activation across mammalian species.

The long lasting Ca^{2+} oscillatory activity during egg fertilisation is a mandatory and sufficient condition to release the egg from its meiotic arrest and support further embryo development. Differences were detected in terms of frequency of occurrence, ranging from one Ca^{2+} spike every few minutes to every hour. These observed Ca^{2+} oscillations in mice last until around the formation of pronuceli, an indication that a plausible termination mechanism is by sequestration into the formed pronuclei. There is undisputed evidence that sperm releases a soluble protein into the egg cytoplasm which triggers the initiation of the Ca^{2+} oscillations. A novel phospholipase (PLCζ) was revealed as the sperm factor after a search into mouse and human testis Expressed Sequence Tag (EST) database. As a confirmation of this theory, injecting sperm extract into mouse eggs resulted in a series of Ca^{2+} oscillations similar to that occurring during fertilisation. When PLCζ cRNA was microinjected into mouse eggs, an indistinguishable Ca^{2+} oscillatory pattern from fertilisation was the result (Saunders et al., 2002). Data from the previous study provided direct and reliable evidence that PLCζ was the long sought after physiological sperm factor. PLCζ gene was found to be existent in a number of mammalian species, moreover, it has been reported that microinjecting chicken PLCζ cRNA into mouse eggs could trigger a series of Ca^{2+} oscillations (Coward et al., 2005). In light of the previous results, a plausible conclusion that a common function for PLCζ across vertebrates may be factual. More recently a new proposition for the sperm factor title has been put forward. Aarabi et al has put forward data claiming that PAWP could be the long sought after sperm factor (Aarabi et al., 2014b). Significantly, they reported that PAWP could cause Ca^{2+} oscillations in mouse and human eggs. However, their case has been challenged by a number of reports. Firstly, a study identifying the SOAF from the perinuclear theca (Perry et al., 1999). The perinuclear theca extracts contained a number of proteins including PLCζ, but not PAWP. Furthermore, no independent research group was able to reproduce Ca^{2+} oscillations after injecting the PAWP protein or the cRNA (Nomikos et al., 2015). The injection of PAWP cRNA showed protein expression levels which exceeded that present in sperm, yet, no Ca^{2+} transients were observed. The final verdict on the PAWP case came when sperm from male mice that were genetically

modified to abolish PAWP. The sperm from those mice successfully triggered Ca^{2+} oscillations and supported development after ICSI in mouse eggs (Satouh et al., 2015). Previous data provides evidence that PAWP cannot be the sperm factor. Therefore, PLCζ remains the only candidate that fulfils the requirements to stand as sperm factor.

Variability in egg sensitivity to PLCζ between species was reported. Hamster eggs were reported to be less sensitive to sperm extracts when compared to mouse eggs (Parrington et al., 1996). The PLCζ protein is thought to be present in an inactive state whilst in the sperm (Swann et al., 2004), upon its release into egg cytoplasm it regains activity suggesting an intrinsic egg mechanism that would allow for the protein switch on. Having that said, differences in the amount of PLCζ, its solubility and activity could subsequently affect the pattern of Ca^{2+} oscillation triggered. Therefore response variability could be a species related difference. Sperm PLCζ is present in variable quantities across species, which could cause a variable response when injected into an egg of another species. It has been shown that pig sperm has higher quantity of PLCζ than mouse sperm (Kurokawa et al., 2005). My results are supported by previous findings, in that mouse eggs demonstrated a significant higher frequency of Ca^{2+} oscillations when microinjected in comparison to those seen in human eggs. Another factor that could explain such a variable response is egg size, cytoplasm volume differs due to size difference between human and mouse eggs. Nevertheless, , I have shown in my results that when the dose of hPLCζ microinjected into mouse eggs is adjusted it can effectively trigger a physiological series of Ca^{2+} oscillations, even though it's an enzyme from another species. Since it has been identified that egg activation occurs within a window of PLCζ concentration (Yu et al., 2008), anything below or beyond that level would have detrimental effects on further embryo development. Therefore, identifying an appropriate amount of hPLCζ to be injected into human eggs would be a cornerstone in making its use in IVF clinics. Unfortunately, my attempts at identifying a dose range for hPLCζ that would induce a Ca^{2+} response similar to that seen during fertilisation in human eggs, have not been successful. My effort was hindered by a number of obstacles. First was the shortfall in the supply of human eggs that were made available for the project. Secondly, was the age of the human eggs that I was microinjecting, all eggs were day 1 post-IVF/ICSI procedure. I would like to bring to attention that the recombinant protein that was used in all of my experiments

was made in our laboratory, the human recombinant protein is tagged to a fusion protein NusA (Nomikos et al., 2013). The choice of the fusion protein was based on its ability to enhance stability, solubility and aid in protein purification (Li et al., 2013). Previous studies from our group revealed that the protein injection devoid from the fusion tag was unable to trigger efficient Ca^{2+} oscillations in mouse eggs. This indicates the importance of the fusion tag in preserving hPLCζ protein function. To test if the tag would have any implications on egg activation and subsequent embryo development, I injected the pure fusion tag into mouse eggs following an ICSI procedure with fresh sperm. Our results indicate that within the range that the protein is injected into mouse eggs it does not hinder the egg's activation or developmental capacity up to the blastocyct stage in mouse embryos. Finally, it is possible that there may be some solubility issues with hPLCζ protein since the mixing with compounds such as PVP lead to a clear precipitation reaction and this is likely to cause protein denaturation.

6.4 Clinical implications of mutational amino acids in PLCζ gene.

Whenever an original amino acid is substituted by another at the germ-line level, it can cause a number of various effects. The effects can range from benign (polymorphism) to a genetic disease with subsequent implications on an individual's health. However, great number of amino acids can be altered without any detrimental effect on the resultant protein functionality (Frillingos et al., 1998). At the other end of the spectrum mutations for certain positions can have a negative impact on protein structure and function. The effect of genetic mal-positioned amino acids will usually be a structural defect within the protein (Yue et al., 2005). In a clinical case following multiple attempts of ICSI with failure to achieve egg activation, a genomic search into the patients PLCζ sequence found evidence for the protein being essential in egg activation. A single point mutation ($PLC\zeta^{H398P}$), made the protein inactive. The amino acid substitution had a helix-destabilising effect on the protein, which led to a disruption of the catalytic Y domain rendering the protein enzymatically inactive in eggs (Heytens et al., 2009). Further confirmation was obtained when the same point mutation was made in mouse PLCζ ($PLC\zeta^{H435P}$). The equivalent PLCζ cRNA with the specific point mutation was microinjected into

mouse eggs and protein expression was monitored. It was reported that Ca^{2+} oscillations were totally abolished despite the fact that protein expression was optimum levels (Nomikos et al., 2011a). Further analysis into the same genome led to a discovery of a second novel point mutation ($PLC\zeta^{H233L}$), replacing a hydrophilic amino acid (Histidine) by a hydrophobic one (Leucine) which had detrimental consequences on protein folding. By microinjecting cRNA encoding the new point mutation into mouse eggs, it triggered an abnormal Ca^{2+} profile. It further reinforced that abnormal PLCζ forms can lead to activation failure in eggs (Kashir et al., 2012).

From that point onwards there has now been increasing evidence that PLCζ can be present as a dysfunctional form in sperm (Kashir et al., 2011, Kashir et al., 2012). The abnormal forms of the protein can contribute to causes of male factor infertility and this supports the rationale behind the use of PLCζ protein as a therapeutic agent for these cases (Yoon et al., 2008, Heytens et al., 2009). For a comparative study, we have developed subtypes of male infertility via heat treatment of mouse sperm to various durations. This resulted in Ca^{2+} profiles that were suboptimal or absent and therefore deficient in causing egg activation and embryo development. After ICSI with sperm that had different time exposure to heat, a second injection with a dose of PLCζ protein was carried out in the eggs. The results presented in this thesis shows that microinjection of PLCζ protein following ICSI has the potential to rescue the eggs from expected activation failure and developmental arrest.

It is not clear how prevalent is PLCζ lack may be in male factor infertility. When the exact pattern of PLCζ in human sperm was investigated no single definite pattern was found, however, different functions were assigned for different localisations (Grasa et al., 2008, Young et al., 2009). A detailed study into the levels and pattern of PLCζ protein in human sperm using immunofluorescent analysis, did not identify any significant difference between patterns from controls that had no history of male factor infertility and sperm from men who had oocyte activation deficiency following IVF (Kashir et al., 2013). They concluded that sperm from normal controls could have a variable level of PLCζ. In reference to that mouse eggs are activated with a range of protein concentration (20-50fg) (Saunders et al., 2002, Yu et al., 2008), it is possible that human eggs have a window range as well for successful egg activation. Yu et al in a previous study showed that successful egg activation per se is not a predictor of successful embryo development, only the eggs that had PLCζ

protein within a specific range managed to further develop post activation (Yu et al., 2008). In an interesting study by Vanden Meerschaut F et al, they used heterologous ICSI to assess the capability of human sperm to trigger Ca^{2+} oscillations in mouse eggs. The results demonstrated that only just over 50% of sperm from controls (normal) had the ability to trigger a response in mouse eggs that could be described as normal if injected into human eggs (Vanden Meerschaut et al., 2013b). Data presented from the previous studies indicate that variable quantities of the protein exist even in "normal" human sperm. To add to the complexity of the situation, currently there is no method to identify the level of protein in sperm prior to its use in IVF. The question for us was, if the recombinant protein was to be used in IVF clinics would it be a matter of patient selection? Should the protein treatment be applied for all patients undergoing IVF treatments?

If PLCζ were to be used for all patients that would potentially mean that some eggs would have an extra dose of the protein on top of a normal sperm. Could that possibly have any consequences on egg activation and subsequent embryo development? To determine whether an extra dose of PLCζ protein to eggs during fertilisation have any toxic effects or block embryo development, a specific experiment was set up. I have injected mouse eggs with a dose of PLCζ protein that is sufficient to support egg activation and embryo development, the microinjection was done post ICSI with fresh sperm. By following the embryos through their development, it was found that the extra protein did not have any negative influence on mouse embryo development up the blastocyst stage. There was no significant difference detected in all stages of embryo development up to the blastocyst stage between eggs activated by one sperm and eggs activated by one sperm plus a dose of PLCζ protein. Furthermore, in a parallel set of experiments I examined the frequency of Ca^{2+} oscillations triggered in both groups: ICSI versus ICSI plus PLCζ. There was no significant difference in the number of Ca^{2+} oscillations detected. My results argued against a previous study that demonstrated a significant difference between Ca^{2+} oscillations triggered in monospermic and dispermic mouse eggs (Faure et al., 1999). However, the differences detected between monospermic and dispermic induced Ca^{2+} oscillations was quite small (5.2+/- 0.3, 6.6+/- 0.3 respectively). Figures are representing the mean number of Ca^{2+} oscillations in each case and their SEM (Faure et al., 1999). The difference between results could be explained by the difference in methodology used in both studies. The study was assessing Ca^{2+} oscillations following an IVF procedure not ICSI. Evidence for the

number of sperm involved in the fertilisation process was assumed based on the number of pronuclei formed (Faure et al., 1999). Moreover, the duration for observing Ca^{2+} oscillations in the previous study was done for two periods of 45minutes with an interruption of 45 minutes in-between while in our study we observed Ca^{2+} oscillations for uninterrupted 2 hour duration. The gap in the imaging process has the potential in missing some of the spikes that could have affected the results. It is imperative to examine the relevance of variability in PLCζ protein detected in human sperm by a direct assessment of its Ca^{2+} release profile in human eggs.

6.5 Response variability to PLCζ microinjection.

Turning away from the sperm as being the main culprit behind the variability seen in the egg's response after fertilisation, the next potential cause could be a factor within the egg. With the advances seen in the fine art of ART, it is obvious that it is a solution for sperm penetration failure. For a successful fertilisation process a wide range of cascades need to take place. The egg quality has an undeniable role in responding to the release of PLCζ from sperm into its cytoplasm. The importance of the level of intracellular Ca^{2+} storage becomes evident once the egg is fertilised. Ca^{2+} brings forward the process of meiosis resumption. Meiosis resumption is an important step in fertilisation, it starts from metaphase to anaphase transition and continues until the formation of the pronuclei (Sen and Caiazza, 2013). The egg is held in a meiotic arrest via the activity of Maturation Promoting Factor (MPF) and MAP kinase (Gonzalez-Garcia et al., 2014). The key event during fertilisation that allows this transition is the periodic Ca^{2+} oscillations, calmodulin dependent protein kinase II (CamKII) is the link between the two events. All the evidence from Ozil's studies suggests that a certain pattern and the size of the Ca^{2+}changes during fertilisation are required for this process to be completed. If the MPF decline has not been suppressed adequately it can regain activity and cause another metaphase arrest. This is supported by the inability of the egg to release the second polar body and form pronuclei if Ca^{2+} oscillation are stopped prematurely (Ozil et al., 2006). That is also what happens in some cases of parthenogenetic activation of eggs with ionophores. As ionophores induce a single Ca^{2+} transient, it is particularly prone to the possibility of not causing a full activation process.

At GVBD the release of nuclear content into the cytoplasm has an associated positive influence on the degree of egg maturation. Yet, some aspects of the maturation process are independent of the nuclear release consequences (Swain and Pool, 2008). Eppig et al 1994 demonstrated that the developmental ability to the blastocyst stage of embryos significantly differed between eggs obtained from mice aged 26 and 18 days. Both groups of eggs morphologically appeared normal as they had released the first polar body. It seemed the younger group were lacking some sort of maternal factors causing a reduced ability to support development (Eppig et al., 1994, Eppig and O'Brien, 1996). Moreover, data from a study investigating the effect of maternal age on eggs and their maturation status, suggests that *in-vitro* maturation is not the cause behind reduced blastocyst developmental rates. Eggs that have matured *in-vitro* taken from 24 day old mice produced Ca^{2+} oscillations in response to fertilisation similar to those matured *in-vivo*. It was suggested that the ability of an egg to generate Ca^{2+} response was developed during the final stages of their development (Cheung et al., 2000). The previous observations give an impression that a simple evaluation by inspecting the appearance of the eggs could be misleading in regard to maturation. Oocyte maturation plays an important role in the redistribution of the ER and the sensitivity of IP3 receptors (Mehlmann et al., 1995, Mehlmann et al., 1996). The maturation process was suggested to cause a build-up in the Ca^{2+} store levels as suggested by Tombes and co-workers (Tombes et al., 1992). However, later it was shown that oocyte maturation enhances IP_3 receptor sensitivity and number, while in terms of Ca^{2+} stores it was suggested a reorganisation process takes place rather than enlarging the reserve levels (Mehlmann and Kline, 1994). The other important aspect of ART, is that it utilises eggs that were forcefully recruited by various ovarian stimulation protocols. Therefore, eggs that were not likely to ovulate naturally become included in IVF treatments and this has the possibly contributing to the causes of fertilisation failure

The presence of Ca^{2+} is an absolute requirement for activation of the Ca^{2+} release mechanism through phosphoinositide specific phospholipase's C. It is well established that Ca^{2+} release during fertilisation is mediated via the hydrolysis of phosphatidylinositol (4,5) bisphosphonate ($PI(4,5)P_2$) and the production of IP_3. Although the main mechanism is the same for all PLC's, the PLCζ has a unique variation. PLCζ does not require any activation upon its entrance into the egg, nor is it in need of G-protein binding to become activated unlike other forms of PLC's. One

possible explanation is its high degree of sensitivity to Ca^{2+}, which is such that once it is inside the egg it is able to hydrolyse $PI(4,5)P_2$ at the basal resting Ca^{2+}level. However, the possibility of other players that mediate PLCζ and $PI(4,5)P_2$ coupling cannot be excluded and are yet to identified. The phosphatidylinositol's (PI) initial production is the endoplasmic reticulum. However, special phospatidyl transfer proteins (PITPs) allow the translocation of the PI into the plasma membrane where it is made into $PI(4,5)P_2$. This translocation process allows for the hydrolysis by PLC's. After hydrolysis, one of the two messengers which is IP_3 diffuses into the cytoplasm and binds to its receptor on the ER triggering Ca^{2+} release (Cox et al., 2002, Swann et al., 2004). The initial release of Ca^{2+} creates a positive feedback loop for further Ca^{2+} release, and the subsequent Ca^{2+} oscillations usually last for several hours (4-6hrs). A series of IP_3 production cycles is the only explanation for the sustainability of the Ca^{2+} oscillations. $PI(4,5)P_2$ is not an egg specific substrate, it is present in most somatic cells. So it could be assumed that PLCζ when injected into somatic cells would trigger a reasonable Ca^{2+} response, but that turned out not to be true. Interesting results were reported when PLCζ was injected into somatic cells (CHO cells) (Phillips et al., 2011). These cells did not show any Ca^{2+} oscillations despite the high expression levels of the PLCζ protein. However, when these cells were microinjected into mouse eggs, Ca^{2+} oscillations were observed (Phillips et al., 2011). Another observation is that PLCζ protein is originally present within the sperm where there is no shortage of $PI(4,5)P_2$, still, the protein remains in its inactive form. That can only lead to one conclusion, which is that the egg has specific factors that can allow the presence of PLCζ to bind to $PI(4,5)P_2$. The intriguing question then would be, by which means can PLCζ distinguish between $PI(4,5)P_2$ present in eggs and that present in other cells? A question, which still has no definitive answer.

It is well known that once PLCζ is released into egg cytoplasm, its main action is to hydrolyse PIP_2. Initially, it was suggested that PLCζ targets plasma membrane PIP_2 (Nomikos et al., 2007). However, a recent study challenged the localisation of PIP_2 on the cell membrane. The data presented revealed that the PIP_2 target for PLCζ is located on intracellular vesicles (Yu et al., 2012). By the use of PLCζ antibodies and immunocytochemistry, PLCζ after microinjection was found localised within the cytoplasm with small vesicles (Yu et al., 2012). The exact nature of the vesicles is unknown. Moreover, the main intrinsic mechanism for this localisation and binding still remains unclear. Nomikos et al in 2007 suggested that the main driving force

behind PLCζ and PIP_2 interaction is through an electrostatic interaction. PLCζ is a positively charged, while PIP_2 is a negatively charged residue (Nomikos et al., 2007). On further investigation it was found that the XY linker region has a preference over the C2 domain in interaction with membrane PIP_2 (Nomikos et al., 2011b). The EF hand was suggested to have the main function of binding to the XY linker (Nomikos et al., 2011b). In a recent study (Escoffier et al., 2016) on a mutation detected in the gene for PLCζ, the homozygous mutation was localised in C2 domain. The C2 domain is known to be a lipid binding protein with little or no affinity for Ca^{2+}. In the case of PLCζ, however, the C2 domain is not necessary for the enzymatic activity of the protein (Kuroda et al., 2006). This suggests the C2 domain may also play some role in targeting. The Escoffier et al study attempted to localize the wild type of C2 mutant PLCz. A significant overlap was identified with the endoplasmic reticulum of the egg in the case of the WT protein, while the protein expressed from the cRNA with the mutation localised around the nucleus. So it is possible that PLCζ also localizes to the endoplamic reticulum.

Of the PLC family, PLCδ1 has the closest homology to PLCζ, the major difference in structure between both PLC's is the absence of the PH domain from PLCζ (Nomikos et al., 2012a) (Nomikos et al., 2012b). The PH domain is responsible for the tethering of PLCδ1 to PIP_2 in the cytoplasmic membrane, interestingly a chimera for PLCζ with the PH domain did not show any greater affinity for the PIP_2 (Theodoridou et al., 2013). Despite the lack of clarity about the exact vesicles that PLCζ protein binds to, and the means by which it binds, it remains clear that PIP_2 hydrolysis is key for generating the Ca^{2+} release profile that supports egg activation and embryo development.

6.6 Ethical and practical considerations in the clinical use of PLCζ recombinant protein.

At the heart of the ethical debate around the IVF domain is whether infertility should be considered as a "disease" and should treatment be regarded as a "cure". Clearly, infertility does not have a direct threat to health in physical terms. Non treatment would not have any immediate impact on public health nor would it cause any rise in the population morbidity/mortality index. However, it has been argued that childlessness has an indirect effect on the well-being of the men and women involved, as well as the social suffering caused in some traditional cultures. There is no doubt that ART carries a significant risk to health as issued in the World Health

Organisation Report in 2001. A staggering 20-fold surge of multiple pregnancy risk has been documented when compared with general population. As a consequence, it is more likely that more preterm infants are born, which is followed by the increased costs of intensive care required for these births. Medical risks for mothers are also a matter of concern. IVF entails invasive procedures which could be life threatening. Multiple pregnancies are not only physically demanding but impose an extra burden financially and emotion on families in the short and long term. Ovarian hyperstimulation syndrome in some of its forms is a life threatening condition (Rizk et al., 1990, Lamazou et al., 2011), and the condition can appear in up to 10% of patients treated with ART (Rizk and Aboulghar, 1991, Joint Society of et al., 2011). Mortality rates of 3 for 100,000 have been reported (O'Donovan et al., 2015, Joint Society of et al., 2011), and the condition can add to the emotional stress if the treatment cycle has to be cancelled (Abuzeid et al., 2003). Despite the wide discussion and debate, currently there is a wide acceptance of treatment modalities in IVF. However, ethically defensive variants have risen due need to solve problems in the field. Sperm, egg and embryo donation generated a great deal of controversy. The freezing process for sperm and embryo, not to mention genetic testing of embryos were all grounds for ethical consideration. Nevertheless, it has been found that medical technology and techniques could be effectively developed when carried out in ethically sound scientific research environment.

Introducing recombinant PLCζ protein, under the existing realm of egg activation for cases of egg activation failure should not need to lead to any reasons in creating controversies over its use. Moreover, the sound evidence of its positive influence in the IVF field will alleviate most of the concerns that will appear. The main aim of this research project was to contribute to the IVF field in an acceptable and ethical way, to enhance the results of fertilisation rates where male factor related failures are reported. In that sense, some of the data presented in this thesis has provided evidence that recombinant PLCζ has the potential to be used as a therapeutic agent to overcome some causes of fertilisation failure.

In the early days of PLCζ discovery, Yoon et al 2008 showed the potential of PLCζ in treating causes of egg activation failure following ICSI. The ICSI procedure was done on mouse eggs, globozoospermic sperm was used and followed by an injection of PLCζ cRNA. The cRNA injection managed to rescue the eggs from activation failure. Ethically this method would not be applicable on human eggs

because the RNA is a genetic material and its injection into human eggs could lead to a genetic modification of the embryo genome after its incorporation (Kashir et al., 2010). Our research team in its early attempts in making the human recombinant PLCζ used a hexa-histidine tag. Results demonstrated a protein expression level that was unsatisfying, however, the protein expressed was functional nut not very active. In a study using the hexa-histidine tag, the level of protein required for inducing Ca^{2+} oscillations was in the range of 5,000fg-10,000fg (Yoon et al., 2012). Furthermore, the expressed protein could not support embryo development to the blastocyst stage (Yoon et al., 2012). In view of the Ca^{2+} release profile of the expressed previous protein along with stability issues, our group turned to a NusA-hexa-histidine fusion preparation that was purified (Nomikos et al., 2013). In contrast to previous tags, a significant level of soluble protein (Nus-A PLCζ) was obtained and it had a molecular mass of about 130KDa as predicted. The 130 molecular mass was a sum of the 70 KDa PLCζ protein and the 60 KDa Nus-A tag (Nomikos et al., 2013). It was established that about 80fg of the protein was able to elicit a series of Ca^{2+} oscillations undistinguishable from those induced by physiological fertilisation in mouse eggs. This level of protein gives an overall increase of protein activity of about 100 times from that of the previous report (Yoon et al., 2012). Furthermore, this protein supported mouse embryo development up to the blastocyst stage (Yu et al., 2008). Our group's research included a closer look into the effects of the Nus-A tag, it was concluded that the tag was both necessary and with no detrimental effects for egg activation and embryo development (Nomikos et al., 2013, Sanusi et al., 2015). Additional confirmation that the protein is a valid treatment for some male factor infertility cases was when PLCζ protein mutations were discovered. When microinjection of the mutant PLCζ into mouse eggs was followed by a rescue injection of the wild type of PLCζ it elicited a series of Ca^{2+} oscillation and supported early embryo development up to the blastocyst stage.

6.7 Practical considerations in introducing PLCζ into clinical settings.

ICSI as a procedure is often taken for granted. However, great care in identifying suitable cases is essential, as there is no significant effect on pregnancy rates for ICSI over IVF. The technique involves a great degree of invasiveness. The sperm deposition into the cytoplasm is not of great concern, but an effect on the disruption

of the cytoplasmic organelles may occur. The multiple step process including egg denudation and plasma membrane piercing using a sharp needle, could hinder developmental success rates. My data is encouraging for the possible use of the protein as a method for rescue of activation failure, or as a general measure during all ICSI procedures. There was no evidence of a harmful effect on embryos developing when normal sperm was used for ICSI with an additional dose of PLCζ. However, with the current method that I was using for the injection protocol it would mean that IVF clinics would need to invest in further equipment to use the protein. To avoid this we carried out a number of experiments testing a suitable method with low pressure injections that could be applicable in IVF clinics in the future. At first we used a specially designed pipette in the laboratory with a bevelled tip mimicking the ICSI pipette but with a narrower opening. I managed after some trial and error to control the amount of material carried over to be injected into the mouse eggs, but the technique would be regarded as a second mechanical injury to the precious human egg and could be avoided if necessary.

The most obvious way to introduce PLCζ into the egg is to do at the same time as the sperm. I initially started by incorporating PLCζ into the injection medium, but due to the influx of Ca^{2+} from the surrounding media (Tesarik and Sousa, 1994) into the eggs alongside PLCζ it produced a significant Ca^{2+} increase in the cytoplasm. The challenging part for me in this experiment was egg survival since it seemed that the eggs were very vulnerable to lysis once injected. This could be explained by the previous reports suggesting that PLCζ is highly sensitive to the level of Ca^{2+} present in solutions (Kouchi et al., 2004). Another possible source of the Ca^{2+} rise during the ICSI procedure is the disruption of the endoplasmic reticulum by the injection pipette (Tesarik et al., 2002) adding to the overall level of Ca^{2+} which can be detrimental to the egg. In an attempt to overcome the low egg survival rate from the previous method, the addition of PLCζ to the sperm drop was tested. The sperm drop is supplemented with PVP which allows the slowdown of sperm to facilitate its pickup. Moreover, PVP allows greater control over the amount of medium injected into the egg during ICSI (Joris et al., 1998). Hence, PVP was supplemented into the sperm drop which aided in the control of the amount of PLCζ being injected while performing ICSI. The method seemed to be reliable in its technique, however, there were aggregates forming in the sperm drop. My initial results regarding the co-injection technique suggest a substantial aggregate formation. The possibility that the aggregates could compromise protein activity or even cause impairment in the

cellular function after being injected needs more evaluation. Additional experiments will be needed to assess any pathogenic effects of the aggregates on egg activation and embryo development. Other sperm slowing agents could be tested in a co-injection technique as a further alternative.

6.8 Safety considerations for the clinical use of PLCζ

In the past few decades there has been a surge in the use of ART, namely IVF and ICSI. Mother and offspring health risks have been noted from the use of such technology. When Bonduelle's group followed a cohort of children born as a result of ART it was deducted that there was an increased likelihood of congenital abnormalities when compared to the cohort conceived normally (Belva et al., 2007). In the previous retrospective analysis the outcome of ICSI procedures was compared to a singleton spontaneous pregnancy. 15/150 of the children in the ICSI group had a major congenital malformation compared to 5/147 in the spontaneous pregnancy group ($P< 0.05$) (Belva et al., 2007). In terms of child development delay, there was no indication that the IVF or ICSI children had any incompetence when compared to naturally conceived children (Bonduelle et al., 2003). On the contrary, the cohort of children born after an IVF/ICSI procedure had a trend that was statistically significant for having a higher IQ and verbal performance (Leunens et al., 2006). However, that could have been a reflection of maternal and paternal educational levels as opposed to that of the spontaneous conception group. Challenges do exist when it comes to reporting risks and adverse outcomes of ART. The main challenge however, is the inconsistency seen in the definition of anomalies identified. What seems to be a set of diagnostic criteria for a paediatrician might not fit into the criteria set by a geneticist. Another aspect is that children born to these procedures might be examined with more vigilance as opposed to children of natural conception and it is true the closer you look- the greater possibility of finding an abnormality. Researchers believe that at least 75% of all infertility has a contributory genetic basis to it and at present limitations exist in identifying all causes. As far as infertility is concerned, the condition itself would limit the spread of any genetic traits to offspring as there will be no offspring. But due to many couples taking on infertility treatment without a complete understanding of the underlying causes, these genetic traits are given the opportunity to resurface.

Existing data suggests, from retrospective studies, that IVF/ICSI is considered to be safe technology (Alukal and Lamb, 2008), but it remains evident that certain birth defects are more common in children born after ART (Hansen and Bower, 2014). Fertilisation to be successful needs to have a specific Ca^{2+} signature (Jones, 1998, Stricker, 1999), physiologically the trigger would be the sperm factor (Saunders et al., 2002, Swann et al., 2004). The effect of this Ca^{2+} signature extends beyond the time frame of their occurrence, and they can have long term effects on late embryo development (Ducibella et al., 2002, Ozil et al., 2005). IVF clinics have been using a number of activation protocols to cover a shortage in the Ca^{2+} signalling system, which is probably due to either a quantitative or qualitative deficiency in the sperm factor. Activation protocols in current practice rely on Ca^{2+} ionophores (Kashir et al., 2010), and these protocols trigger a single Ca^{2+} rise within the egg. It is suggested that both ionomycin and A23187 cause a Ca^{2+} increase by increasing plasma membrane permeability to Ca^{2+} influx. It was suggested as well that they might have an effect on internal Ca^{2+} stores through a similar mechanism as IP3 induced Ca^{2+} release (Yoshida and Plant, 1992). In a study by Heindrycks's group, it was shown that ionomycin was superior in triggering a larger Ca^{2+} transient than A23187 in mouse eggs and in vitro matured human eggs (Nikiforaki et al., 2016). However, it could be argued that the difference seen in activation rates was due to protocol differences, as eggs were exposed to a double exposure to ionomycin while the A23187 group had only a single exposure. Again the total duration that the eggs were exposed to both ionophores was different giving another explanation for the differences detected in the total Ca^{2+} release profile for both ionophores. From the early use of parthenogenetic activation there were reports indicating that they could have effects on developing embryos. It was reported that mouse eggs activated parthenogenetically had an embryo size that was significantly smaller than that of normal fertilisation (Hardy and Handyside, 1996). It was suggested that the effect could extend to the early post-implantation period were mouse embryos had a diminished developmental capability possibly due to a defective gene expression (Surani and Barton, 1983). Having said that, other research groups have reported results that disagree with the notion that parthenogenetic activation has deleterious effects on future embryo development (Loi et al., 1998). With all the controversy in regard with safety concerns with the use of chemicals to induce artificial oocyte activation, there are many reports describing their use and valuable outcome on human eggs (Mansour et al., 2009, Nasr-Esfahani et al., 2010, Borges Jr et al.,

2009b, Borges Jr et al., 2009a). It has to be said that up to date there has been no reports for any mental or physical side effects for children born after assisted activation protocols were applied (Kyono et al., 2008, Nasr-Esfahani et al., 2008). It is also an issue that all of the activation protocols reported had no parallel control groups to compare to.

J.P Ozil and co-workers conducted a study to determine if interfering with the Ca^{2+} release profile during fertilisation would have any compromising effect on embryo development. The experiments were conducted on mouse eggs that have fertilised *in vivo,* and the interference with the physiological Ca^{2+} profile was either by premature termination of the Ca^{2+} oscillations or overriding the endogenous response by triggering a series of 20 Ca^{2+} oscillations. Embryos resulting from both protocols were returned to recipients to minimise detrimental effects of in vitro culture on gene expression and development. Results have shown that although blastocyst developmental rates were not compromised for both groups, it was evident that interfering with the physiological Ca^{2+} signal had detrimental effects on further developmental potential of the blastocysts. The consequence of having few Ca^{2+} oscillations was seen as having less offspring born, indicating that the implantation process was compromised. Whereas in the case of triggering a higher Ca^{2+} oscillation frequency pattern it was found that there was a greater variability in the weight of offspring (Ozil et al., 2006). My results tend to disagree slightly with these previous finding as I have shown that by reducing the number of Ca^{2+} spikes during early fertilisation it does significantly affect the rate of blastocyst developing in mouse embryos. However, the differences could be due to the strain of mice used, since previous studies by Ozil used F1 hybrid mice which are genetically selected to develop to blastocyst at high rates, whereas I used an inbred strain of mice with ~50% development rate to blastocysts stages. Still, both results highlight the influence of perturbing the physiological Ca^{2+} profile. The influence of a diminished Ca^{2+} profile was seen as reduced capability for blastocysts to implant, while blastocysts developing from the overstimulated Ca^{2+} response were failing to continue development post implantation (Ozil et al., 2006). In light of previous findings, it is of a concern that performing ICSI to treat male infertility would allow introduction of a sperm into the egg that cannot trigger the required set of Ca^{2+} oscillations. That will ultimately lead to failure of implantation or failure to develop to term as in the case of early miscarriages. These pre-implantation and post-implantation failures should not necessarily be seen as a poor outcome because

they could be seen as an alternative to yielding an offspring with certain diseases. A kind of natural selection process may occur where only the most viable embryo can continue to develop.

In previous studies our group has shown that PLCζ protein can activate and support development up the blastocyst stage in human and mouse eggs (Cox et al., 2002, Rogers et al., 2004). However, the amount of protein that would support early embryo development was not clarified. It was until Yu et al demonstrated a relationship between the amount of protein injected and the developmental ability of mouse embryos (Yu et al., 2008). The study revealed that the egg activation process is sensitive to the level/frequency of Ca^{2+} oscillations. However, the ability to proceed to the blastocyst stage had a "window" requirement for both the amount of protein and ultimately the frequency of Ca^{2+} oscillations. The 'window' covers about a fourfold range of PLCζ concentrations. The results in the previous study support my findings, where I found that there was a significant difference between the number of Ca^{2+} spikes occurring in embryos that have progressed to the morula stage and the ones arresting at the 2-cell stage. In Yu's et al study it was found that low expression levels of protein led to a poor Ca^{2+} pattern, whereas a high protein level had a corresponding high Ca^{2+} oscillation frequency and both had poor blastocyst development rates. From our previous studies, we can assume that whenever PLCζ protein is microinjected into mouse eggs if not within the "window requirement" the embryos will not be developing. It can be generally concluded that far too much or too little of the protein would translate into failure of embryo development. Furthermore, if to extrapolate the findings from studies on mouse eggs to human eggs then our results may suggest a self-limiting safety mechanism for PLCζ protein since embryos receiving too much will not develop further than the cleavage stages. This makes PLCζ protein a very strong prospect for treating its deficiency that can be held accountable for some of the failed fertilisation failure after ICSI (Battaglia et al., 1997, Heindryckx et al., 2005). Safety concerns over any new treatment protocol are a justifiable matter and it should be in the hands of patients to make informed choices. Making an informed decision can only happen when proper counselling about all options is done. All couples seeking treatment for infertility should receive information for their treatment options before they start the long stressful and emotional journey. Patients should always be reminded that there is no guarantee for perfect outcomes although the scientific evidence of harm is

negligible. However, they should also be reminded that even in the case of couples with normal fertility, no such guarantee exists.

6.9 HyPer probe and limitations in eggs.

It is established that abnormal patterns of Ca^{2+} oscillations during early events of fertilisation can influence embryo development capacity (Ozil et al., 2006, Yu et al., 2008). More specifically the high frequency of Ca^{2+} oscillations causes failure of post-implantation development. However, the mechanism and the signalling pathway for a detrimental outcome are yet to be known. The more interesting question is by which means can an early phenomenon during fertilisation influence such a late event in embryo development? What mediates the developmental arrest? It is tempting to speculate that a link between the frequency of Ca^{2+} oscillations during fertilisation and the ROS production level has a cause and effect relationship. A speculation that a defective embryo development is caused by an increasing ROS level due to a high Ca^{2+} oscillation frequency during fertilisation. In aged mouse eggs it is known that the Ca^{2+} release profile will switch from a fertilisation signal into an apoptotic triggering signal (Gordo et al., 2000). The suggested mechanism behind this is the loss of the anti-apoptotic gene balance which determines whether a cell lives or dies (Elmore, 2007). However, in freshly ovulated eggs the signalling system for developmental arrest remains unclear. A cellular reaction to an overdose of any signalling stimulus would be shown as cell toxicity or cell death. Ca^{2+} is an example of such a stimulus, that whenever rises beyond physiological levels can cause cell death (Trump and Berezesky, 1996).

It is well established that the mitochondria population within the egg is the only source of future mitochondria in all cells for the developing embryo (Jansen, 2000). The multifunctional organelle is responsible for energy production (ATP) and aids in the balance of cytosolic Ca^{2+} levels by acting as a store for excess levels. Another aspect of its many functions is that it acts as a store for proapoptotic factors and a source of ROS production in the embryo (Minamikawa et al., 1999). Some of the developmental compromise could be attributed to the sensitivity of this particular organelle to insults during in vitro manipulation/fertilisation (Thouas et al., 2004). Such insults can result in ROS production from the mitochondria which damages organelles where after they leak into the cytoplasm triggering a degenerative cascade (Jansen, 2000).

Mitochondria produce both ROS and ATP. It has been known for some time that the morphological appearance of some mammalian species such as bovine eggs is directly correlated with the level of ATP within. Moreover, the developmental capacity to the blastocyst stage is dependent on ATP (Stojkovic et al., 2001). Furthermore, in other reports it has been suggested that for successful human and mouse embryos development there is a mandatory requirement for a certain level of ATP (Ginsberg and Hillman, 1973, Van Blerkom et al., 1995, Van Blerkom, 2011). It is known that mouse pre-implantation embryos rely on pyruvate as an energy source due to the block in the glycolysis pathway (Ray et al., 1995). In a more recent study it was observed that during mouse embryo fertilisation, in particular during the Ca^{2+} oscillatory phase, there was evidence for an increase in the production of ATP. The ATP increase was from mitochondria and was both necessary and sufficient for embryo development (Campbell and Swann, 2006). The previous study demonstrates that the Ca^{2+} oscillations influences the influx of Ca^{2+} into mitochondria and subsequently modulating ATP production levels to meet the embryo requirement during fertilisation. However, it has to be shown whether the temporary increased levels of ATP has any clear effect on embryo development (Quinn and Wales, 1973). Moreover, an ATP dependant protease (proteasome) in cells has been found to respond to low and high levels of ATP. The bidirectional effect of the ATP levels on this enzyme has detrimental effects on cell viability (Huang et al., 2010).

A key molecule for intracellular signalling is without doubt ROS, and H_2O_2 is one of the most important species. The mitochondria are a major source for the ROS, within the cell they have a wide spectrum of influence, from cell proliferation to cell death. They are as well involved in the activation process of a number of genes and contribute to signalling pathways (Turpaev, 2002). The source of ROS in an egg during fertilisation can be either from the mitochondria during the oxidative phosphorylation process or from the cytoplasm via cytoplasmic oxidases (Nohl, 1994). The ability of the mitochondria to uptake Ca^{2+} during fertilisation into its matrix, raises a question of what impact would that have on the egg activation process. The developmental capacity of the mammalian embryo is affected at various levels by any imbalance in the antioxidant system (Guérin et al., 2001, Morado et al., 2009). A number of reports have linked the detrimental influence of increase levels of in vitro ROS production on pre-implantation embryo development (Guérin et al., 2001, Yang et al., 1998, Bain et al., 2011). In human embryos there

was a direct link found between the internal levels of H_2O_2 and apoptosis (Yang et al., 1998). Thompson JG's group reported an observation of an increase in the production of ROS during early embryo development in bovine eggs (Lopes et al., 2010).The observation was based on the first cell division in bovine embryos, it was found that the first cleavage was preceded by an increase in mitochondrial activity. This rise in mitochondrial activity is required to meet the energy demand necessary for the cleavage to complete. In addition to the mitochondrial activity, there was an increase in the oxygen consumption levels. Furthermore, an oxygen consumption peak was detected on two separate incidents post fertilisation within the first 24hrs in bovine and sea urchin eggs (Heinecke and Shapiro, 1992, Lopes et al., 2010), these peaks had parallel rises in the production of ROS level. These data all indicate a possible link between mitochondrial activity and the overall ROS levels. During the early stages of mouse embryo development it was shown that during pronuclei formation there was an increase in glucose oxidation through the pentose phosphate pathway (Pantaleon et al., 2001). Moreover, reports support the observation that around the time of fertilisation in mouse eggs there is a respiratory burst that directly causes a measurable increase in the intracellular levels of H_2O_2 (Nasr-Esfahani and Johnson, 1991).

All of all, the literature suggests that mitochondrial activity could be one of the causes behind a rise in the ROS level during fertilisation. The level of ROS if beyond the egg's ability to handle, could possibly explain some of embryo developmental failure. In the third chapter of my results, I have attempted to unravel the mechanism of developmental failure observed in certain patterns of Ca^{2+} oscillations, especially the high frequency pattern. In light of previous reports, the hypothesis was that an early event during fertilisation could negatively influence embryo development. The hypothesis was that high Ca^{2+} oscillation patterns trigger an excess production of ROS via its effect on mitochondria. I attempted this study with the aid of a new probe used for detection of H_2O_2.The claim was that the probe was superior to other previous probes in monitoring H_2O_2 changes *in vitro* (Belousov et al., 2006). The author of the previous paper, pointed to the various pitfalls of the widely used ROS indicator utilised in the majority of studies investigating ROS and its effect during fertilisation. It was argued that the dichlorofluorescein (DCF) derived indicator had an unspecific reaction to H_2O_2, as it could react to the oxidisation of other species. The more significant drawback was that the indicator itself could contribute to the amplification of the fluorescent signal. The DCF indicator, upon exposure to light,

can cause artificial generation of ROS and cause a signal exaggeration. The new HyPer probe constructed was claimed to have high specificity towards H_2O_2 and not other oxidants. Initially in our experiments it was found that the probe introduced into mouse eggs indeed responded to an increase in H_2O_2 levels in the surrounding media. However, doubts casted by the changes detected in the fluorescent signal seen during fertilisation, indicated that there could be other contributors to the signal change. Upon further examination, it was found that the fluorescent changes detected were coinciding with intracellular pH changes within the egg. The change in the egg intracellular pH was investigated, but unfortunately the source was unidentified and its prevention was unsuccessful. The probe has been criticized for its sensitivity to pH changes (Muller, 2009, Quatresous et al., 2012), and since then other enhanced versions of the probe have been developed named HyPer2 and HyPer3 (Markvicheva et al., 2011, Bilan et al., 2013). The group is claiming that the new probes have a reduced pH sensitivity, however, further improvements were recommended (Ermakova et al., 2014, Lukyanov and Belousov, 2014). It would be interesting to examine the new probes in mouse eggs and identify if any improvements will be seen in its pH sensitivity. Unfortunately, due to time constraints of my study, further enquiry would need to be set up as a future project.

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