IVF based approaches towards the treatment and prevention of mitochondrial disease

Laura Irving BSc MSc

Supervisors

Professor D. M. Turnbull

Professor M. Herbert

Institute of Ageing and Health

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Abstract

Mitochondria are strictly maternally inherited, with all paternal mitochondria being destroyed following fertilisation. Women known to be carriers of pathogenic mtDNA mutations are therefore at increased risk of conceiving affected children. These women are currently offered the following options to aid in genetic counselling: oocyte donation, prenatal genetic diagnosis (PND) or preimplantation genetic diagnosis (PGD).

One of the aims of this thesis, was to examine the feasibility of PGD for mtDNA inherited disorders, with specific emphasis on answering the following questions: how accurately does the mutation load observed in the biopsied blastomeres reflect the mutation load in the remaining embryo, are those mutation loads initially observed in the biopsied blastomeres maintained throughout preimplantation embryonic development and do mutation loads observed in the inner cell mass reflect those mutation loads observed in the extra-embryonic trophectoderm cells? In my thesis, I have now been able to provide data towards answering each of these questions through the examination of mutation loads in oocytes, embryos and blastocysts obtained from mitochondrial patients undergoing fertility treatment.

Techniques, which have been developed in my current laboratory, have facilitated the characterisation of a nuclear transfer technique known as pronuclear transfer (PNT). This is a method to prevent the transmission of mitochondrial DNA disease from mother to child (Craven et al, 2010). As part of the work for my thesis, I have examined the reproducibility of the PNT technique by assessing whether the procedure could be performed by different operators, whilst maintaining levels of efficiency, survival and developmental outcome.

Experiments are now being performed to examine the feasibility of PNT in normally fertilised human zygotes, created from donated oocytes. As it is unlikely that egg collection will be possible from two independent donors on the same day, the final purpose of this study was to examine the potential and feasibility of vitrification of eggs or fertilised embryos at both the pronuclear (PN) and Metaphase II (MII) stage for the purpose of the PNT technique.

In summary, my studies has examined the reliability of current methods to reduce the likelihood of having a child affected by a mitochondrial DNA disorder and new techniques currently being developed to prevent the transmission of defective mitochondrial DNA, altogether. I hope this will provide fresh hope for patients with mitochondrial DNA disease.

Acknowledgements

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Presentations

- **North east postgraduate conference** September 2011 (Oral presentation)
- **North east postgraduate conference** September 2010 (Oral presentation)
- **Neuromuscular Dystrophy Campaign translational research conference** (NMD) March 2011- Poster presentation
- **Preimplantation genetic diagnosis international society (PGD-IS) conference** May 2012 (Chosen to give oral presentation).
- **IAH/IHS joint research symposium** March 2013 (Poster presentation) – Won prize for best poster

Publications

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Abbreviations

ADP	Adenosine diphosphate
APC	Anaphase promoting complex
APS	Ammonium persulfate
ATP	Adenosine triphosphate
CO_2	Carbon dioxide
CPEO	Chronic progressive external opthalmoplegia
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytodineine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dH_20	Deionised water
DIC	Differential interference contrast
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleic triphosphate
DOA	Dominant optic atrophy
dpc	Days *post coitum*
ds	Double stranded
EDTA	Ethylenediaminetetreacetic acid
ER	Endoplasmic reticulum
ES	Equilibration solution
FSH	Follicle stimulating hormone
GFP	Green fluorescent protein
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotrophin
HCl	Hydrochloric acid
HFEA	Human Fertilisation and Embryology Authority
HSA	Human serum albumin
HSP	Heavy strand promoter
H-strand	Heavy strand
HVJ	Haemagluttinating Virus of Japan
HVJ-E	Haemagluttinating Virus of Japan envelope
ICM	Inner cell mass

ICSI	Intracytoplasmic sperm injection
IU	Intravenous units
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
kb	Kilobase
KSS	Kearn's Sayre syndrome
kV	kilovolts
LHC	Last hot cycle
LOHN	Leber's hereditary optic neuropathy
LSP	Light strand promoter
L-strand	Light strand
M	Molar
MELAS	Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
MERFF	Myoclonic epilepsy and ragged red fibres
Met	Methionine
Met I	Metaphase I
Met II	Metaphase II
Mfn	Mitofusin
MILS	Maternally inherited Leigh syndrome
ml	Millilitre
mM	Millimolar
mm	Millimetre
MPF	Maturation promoting factor
mRNA	Messenger RNA
ms	Milliseconds
MST	Maternal Spindle Transfer
mtDNA	Mitochondrial DNA
mtEF	Mitochondrial elongation factor
mtIF	Mitochondrial initiation factor
mtSSB	Mitochondrial single-stranded binding protein
nM	Nanomolar
NaOAC	Sodium acetate
NaOH	Sodium hydroxide
NT	Nuclear transfer
OH	Origin of heavy strand replication

OL	Origin of light strand replication
OXPHOS	Oxidative phosphorylation
PB	Polar body
PBB	Polar body biopsy
PBS	Phosphase buffered saline
PCR	Polymerase chain reaction
PCS	Preimplantation genetic screening
PFA	Paraformaldehyde
PGC	Primordial germ cell
PGD	Preimplantation genetic diagnosis
PMSG	Pregnant mare's serum gonadotrophin
PND	Prenatal genetic diagnosis
PN	Pronucleus
PNT	Pronucelar Transfer
POLG	Polymerase gamma
POLRMT	Mitochondrial RNA polymerase
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
ROS	Reactive oxygen speciesrRNA Ribosomal RNA
SD	Standard deviation
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TCN	Total cell number
TE	Trophectoderm
TEMED	N,N,N'N'-tetramethylenediamine
TFAM	Mitochondrial transcription factor A
TFB	Transcription factor B
TMRE	Tetramethylrhodamine ethyl ester
tRNA	Transfer RNA
UV	Ultraviolet
V	Volts
VS	Vitrification solution
ZP	Zona pellucida

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Chapter 1

Introduction

Chapter 1: Introduction

1.1 Mitochondrial Biology

1.1.1 Basic Mitochondrial Biology

A major function of mitochondria is to support aerobic respiration and generate energy substrates in the form of ATP to support intracellular metabolic pathways (Schapira, 2006). Here a series of enzymes catalyse the transfer of electrons along a series of electron carriers to a final electron acceptor (molecular oxygen, which is subsequently reduced to water) eventually culminating in the generation of ATP.

Mitochondria have also been shown to have additional functional roles within the cell forming a complex branching network with other organelles rendering them as essential components required for normal cellular function. Given the fundamental role of mitochondria in the body, defects affecting their function can have several profound outcomes (Schapira, 2006).

Mitochondrial diseases are generally attributed as a group of disorders which result from malfunctioning mitochondria. Until relatively recently, their prevalence in the population has been relatively underestimated.

1.1.2 Mitochondrial origins

Mitochondria were once free living life-forms derived from the ancient α – proteobacteria, which were taken up by the 'host' cells as endosymbionts and transformed into intracellular organelles (Reyes-Prieto, 2007; Gross and Bhattacharya, 2009; Alcock et al, 2010). The exact mechanism by which mitochondria were engulfed has yet to be fully elucidated. Several hypotheses have been proposed to attempt to explain how this might have occurred (Martin, 2010; Embley and Martin, 2006). The most well, established of these assumes that mitochondria originated following engulfment via phagocytosis by an α-proteobacterium by an anaerobic organism (Martin, 2010). The alternative hypothesis posits that mitochondria originated following the simultaneous fusion of a methanogenic Archaebacterium (host) with a

hydrogen producing proteobacterium (symbiont) (Gray et al, 1999) (Fig1.1). However as a consequence of endosymbiosis and evolutionary pressure the mitochondrial genome gradually relinquished more of its genomic content, resulting in the transfer of genes to the nuclear genome (Roger, 1999; Embley and Martin 2006). However mitochondria retained a total of 37 genes encoding 2 rRNA, 22 tRNA and 13 polypeptides. Why these genes have not undergone transfer to the nuclear genome has and still is fiercely debated. One hypothesis proposes that some proteins are too highly hydrophobic to be imported across the mitochondrial membranes (Papot and Vitry, 1990). This may also be coupled to the fact that very hydrophobic proteins might also be mis-routed or fail to reach their end targets. A second hypothesis suggested for the retention of genes in the mitochondrion is the potentially toxic nature of the gene products if present within the cytosol (Martin and Schnarrenberger, 1997). The third considers that genes whose products have key roles in the electron transport and energy coupling must be quickly and directly regulated by the redox state of the mitochondrion (Allen, 1993; Adams and Palmer, 2002). It has also been suggested that the non-standard genetic code used in the mitochondrion of many eukaryotes is also involved in restricting complete transfer of all genes to the nuclear genome (Adams and Palmer, 2002).

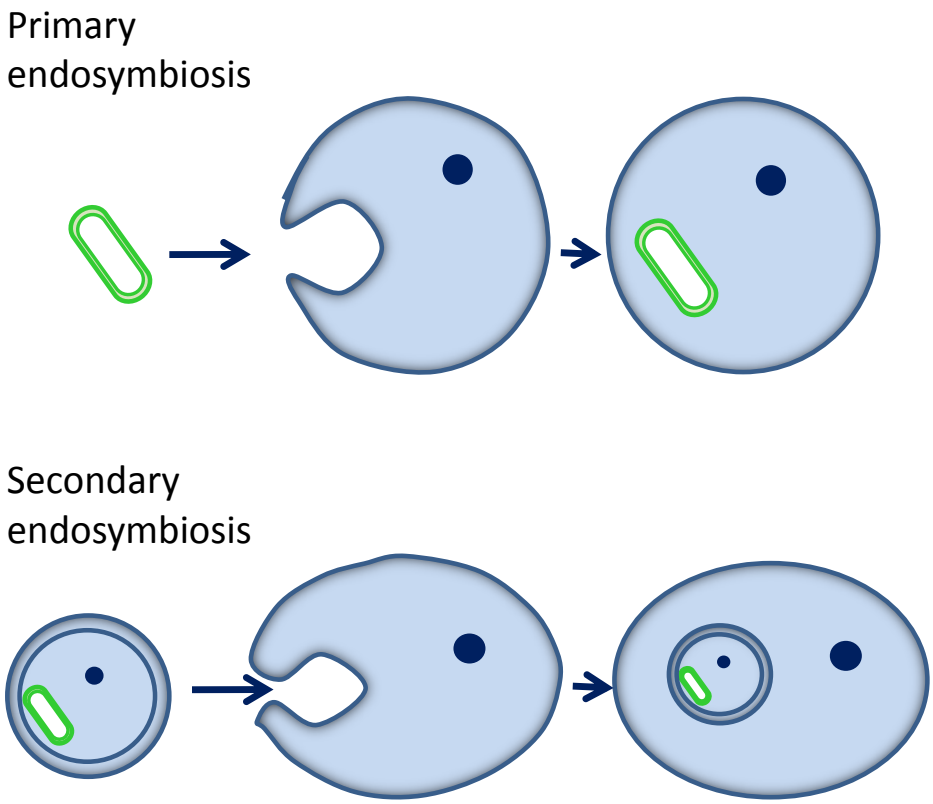


Figure1. 1Primary and secondary endosymbiosis

The above schematic diagram represents the two models proposed to explain the mitochondrial endosymbiosis theory. (a) Primary endosymbiosis was said to involve the engulfment of a bacterium (α-proteobacteria) by another free-living organism. (b) Secondary endosymbiosis is said to have occurred when the product of primary endosymbiosis was engulfed and retained by another free-living eukaryote. An essential part of secondary endosymbiosis has been the transfer of genes for plastid proteins from the endosymbiont to the host nucleus (Adapted from original taken from Embley and Martin, 2006).

1.1.3 Mitochondrial Structure

Mitochondria are unique organelles, fundamentally required by the cell in order to transduce energy, and are approximately 0.5-1μm in diameter and up to 7μm long. Their shape and number is heavily dependent upon the particular tissue they reside. Mitochondrial shape can also vary where they have been observed as spheres, rods and filamentous bodies (Campello et al, 2010). The number, distribution and morphology of mitochondria, often heavily influenced by the cells function and overall energy demands.

It is also increasingly apparent that during early embryo-foetal development mitochondrial structure and dynamics are considerably different to those observed in adult cells and tissues where the morphology of cristae within mitochondria appears relatively underdeveloped and sparse within the sphere-like mitochondria (Squirrel and Bavister, 2000; Motta, 2000). Indeed it is not until the latter stage of preimplantation development due to an increase in the energy demands that the density of inner mitochondrial membrane folding increases in response (Sathananthan and Trounson, 2000) (Figure1.2b).

Initial observations concerning mitochondrial structure were primarily made through electron microscopy work (EM), which ultimately led to the proposal of two models of predicted mitochondrial structure (Palade, 1953 and Sjostrand, 1953). The two models initially proposed were named the ‘septa’ model (Sjorstand, 1953) and ‘baffle’ model (Palade, 1953), with the ‘baffle’ model ultimately being accepted and now depicted in most text books as the perceived model for mitochondria. This model included the mitochondrial double membrane, both an inner and outer membrane, which define two internal compartments; the intermembrane space, which is the area that lies between the two membranes and the matrix, which lies within the inner membrane. The inner membrane is further highly convoluted with numerous folds, which give rise to the ‘cristae’ a feature highly characteristic of mitochondria. Cristae have a functional role within the mitochondria where they significantly increase the surface area on which ATP synthesis can occur, therefore increasing the energy tranduction efficiency of the organelle. The space within the cristae folds is sometimes referred to as the ‘intracristal space, where relatively recently new evidence, driven by the novel process of

cryoelectron tomography, has revealed this space to be a unique environment (Perkins and Frey, 2000; Frey and Mannella, 2000)

The outer membrane contains many porin molecules (voltage-dependent anion channel or VDAC), a type of transport protein that forms large aqueous channels through the lipid bilayer (Alberts *et al*, 2005). The outer membrane is therefore rather like a 'sieve' as it is permeable to molecules of 5000 daltons or less, including small proteins. However once a molecule accesses the inter-membrane space, it is normally unable to pass through the inner membrane, which is impermeable in comparison to the outer membrane.

The inner membrane is highly specialised in comparison to the outer membrane and the primary site of the electron transport chain (ETC). Furthermore it contains proteins with three different types of functions; those that carry out the oxidation reactions of the electron-transport chain; the actual ATP synthase that is responsible for making ATP and thirdly transport proteins which facilitate the passage of metabolites into and out of the matrix (Alberts, et al, 2005)(Fig1.2a).

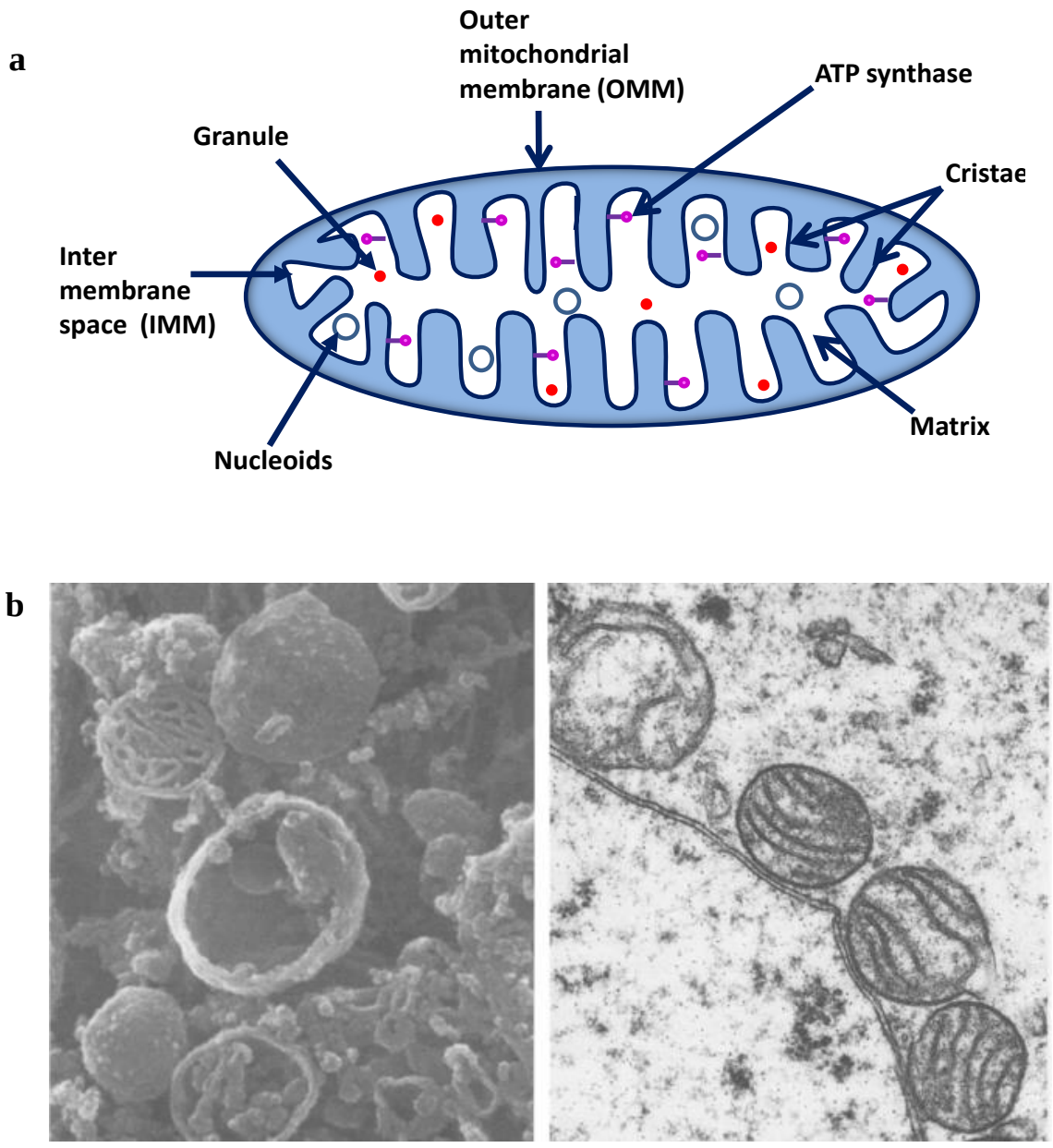


Figure1. 2 Mitochondria within oocytes

(a) Schematic representation depicting the traditional morphological structure of mitochondria. (b) Electron microscopy (EM) images depicting mitochondria found within human oocytes and early cleavage stage embryos. These mitochondria appear significantly different morphologically and structurally to mitochondria found at any other stage of development. The organelles are sphere –like and contain fewer cristae, if indeed they are present at all. This is likely to be due to the reduced energy demands at this stage of development (Images taken from Motta et al, 2000).

1.1.4 The mitochondrial respiratory chain

The electron transport chain comprises four complexes through which electrons are passed sequentially until they reach the final electron acceptor. During electron transport, the enzymes of the electron transport chain create a proton gradient across the inner mitochondrial membrane. This gradient is then utilised by the enzyme FoF1 ATP synthase to produce ATP an extremely important molecule which is often referred to as the 'energy currency of a cell' (as depicted in Fig1.3).

Each respiratory chain complex is composed of multiple subunits, most of which are encoded by the nuclear genome, including all of the subunits of complex II (Morris et al, 1995). The remaining four complexes comprise of both mitochondrial and nuclear encoded subunits. Respiratory chain deficiencies are frequent causes of disease and result in a wide variety of diseases phenotypes (Morris et al, 1995).

1.1.4.1 Complex I (NADH-coenzyme Q oxidoreductase)

Complex I is the first and by far the largest and most complex protein in the electron transport chain It is composed of 46 subunits (Yagi and Matsuno-Yagi, 2003; Carroll et al, 2006a). A total of seven subunits are encoded by mitochondrial genome and the remaining 39 by the nuclear genome (Hirst et al, 2003; Papa et al, 2007). This enzyme catalyzes the electron transfer from NADH to ubiquinone, which is subsequently linked to the translocation of four protons through the inner mitochondrial membrane (Torres-Bacete et al, 2011)

1.1.4.2 Complex II (Succinate-Q oxioreductase)

Complex II consists of four protein subunits (SDHA, SDHB, SDHC and SDHD), contains a bound flavin adenine nucleotide (FAD) cofactor, three iron-sulphur clusters [2Fe-4S], [3Fe-4S], and [4Fe-4S] and a heme group. Complex II is the entry point of the electron transport chain for $FADH_2$ formed as part of the citric acid cycle during the oxidation of succinate to fumarate by succinate dehydrogenase (Sun et al, 2005). Succinate dehydrogenase also forms part of the succinate-CoQ oxidoreductase complex otherwise known as complex II. The two electrons are then transferred from $FADH_2$ to

a series of iron-sulphur clusters which finally pass to ubiquinone (Q) and through a redox reaction form ubiquinol (QH_2).

1.1.4.3 Complex III (Q-cytochrome c oxidoreductase)

Complex III was first discovered and purified from bovine heart mitochondria in 1961 (Hatefi et al, 1961; Hatefi et al, 1962). In mammals the enzyme is a dimer, with each of these sub-complexes containing 11 protein subunits, an iron-sulphur cluster and three heme prosthetic groups and two cytochromes (Iwata et al, 1998).

The function of ubiquinol-cytochrome *c* oxidoreductase is to catalyze electron transfer from dihydroubiquinone (QH_2) to oxidized *cytochrome c*, (Cyt *c*) which is subsequently coupled to transmembrane proton translocation (Leung and Hinkle, 1975). The Q-cycle, refers to the sequential oxidation and reduction of the lipophilic electron carrier, coenzyme Q10, whose role in the pumping of protons across the lipid bilayer, and was initially proposed by Peter Mitchell (1976). Here two electrons are transferred from QH_2 to two molecules of cytochrome *c* (a water soluble electron carrier) as cytochrome *c* is only able to accept one electron at a time. Two QH2 molecules bind to the complex consecutively, each giving up two electrons and two H^+. The protons are released to the cytosolic side of the membrane. The first QH_2 binds to the complex (via the Q binding site Q_o) n and its electrons travel through the complex, to two different locations. The first electron flows through the complex to its target oxidized cytochrome c, and converting it to its reduced form. The second electron passes to an oxidized ubiquinone (found in a second Q binding site Q_i). Here the quinone in the first binding site is reduced to semi-quinone radical anion ($Q.^-$) following the transfer of the first electron. The ubiquinione now fully oxidized leaves. A second molecule of QH_2 then binds and the first electron is passed to cytochrome *c* where it then reacts' the same as the first time an electron was received. The second electron passes to the partly reduced ubiquinone and subsequently takes up two protons from the matrix to form QH_2. The removal of two protons from the matrix leads to the formation of a proton gradient (Berg, Tymocozko and Stryer, 2012).

1.1.4.4 Complex IV (Cytochrome c oxidase)

Complex IV is the final protein complex in the electron transport chain (Calhoun *et al*, 1994) and transfers electrons to oxygen, while pumping protons across the membrane (Yoshikawa et al, 2006). The mammalian complex IV contains 13 subunits, two heme groups in addition to multiple metal cofactors (Tsukihara et al, 1996). Complex IV catalyzes the transfer of electrons from the reduced form of cytochrome *c* to molecular oxygen, the final acceptor of the chain. Furthermore it is coupled to the transfer of four protons across the membrane, contributing to the proton gradient.

1.1.4.5 FoF1ATP synthase

The mitochondrial FoF1 adenosine triphosphate synthase (FoF1 ATP synthase) is a membrane bound enzyme that couples the proton motive force across the inner mitochondrial membrane (IMM) to the synthesis of ATP in the matrix (Boyer, 1997; Walker, 1998; Rubinstein et al, 2003). Electron microscopy, crystallographic and biochemical studies have all played pivotal roles in elucidating the function of the FoF1 ATP synthase. These studies have revealed that ATP synthase is a large complex which is composed of a proton-conducting unit and a catalytic unit (Berg et al, 2002). These two subunits are more often referred to as the F_1 and F_0 subunits. The majority of the F_0 subunit is embedded in the inner mitochondrial membrane; this is highly hydrophobic and contains the proton channel. The F_1 'ball shaped' subunit protrudes into the mitochondrial matrix and is responsible for the catalytic activity of the synthase. These two subunits are then connected via 'the central γε stalk' and an exterior column (Berg, Tymoczko and Stryer, 2012) (Fig1.3).

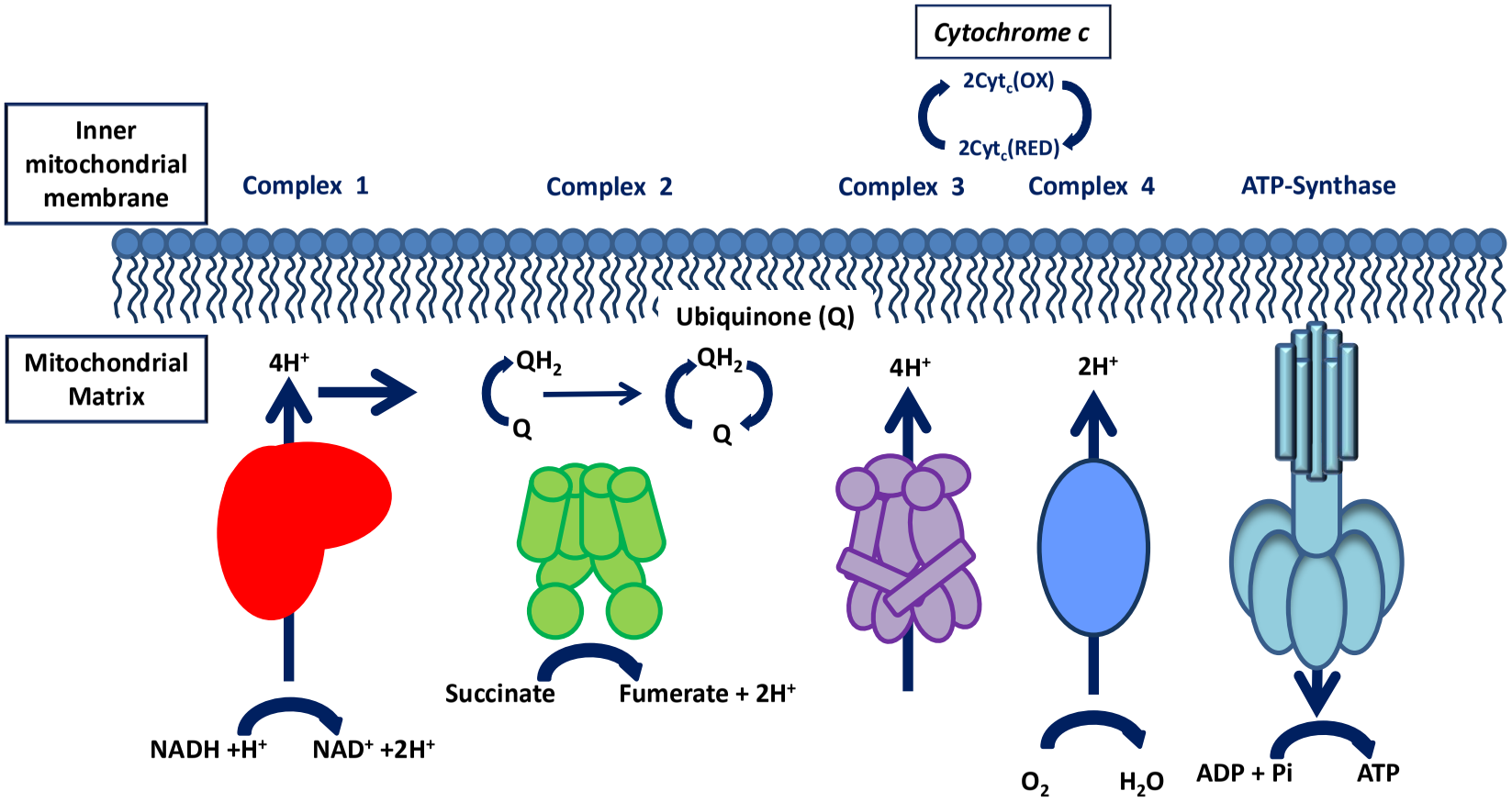


Figure1. 3The mitochondrial respiratory chain

Above diagram depicts the electron transport chain and the movement of electrons between the four electron carriers which drives the exchange of hydrogen ions across the mitochondrial membrane and ultimately results in the establishment of a proton gradient and product of ATP. Adapted from original image by Yong-Ling et al, 2008.

1.2 Mitochondrial DNA

1.2.1 Mitochondrial genome

Mitochondria are unique organelles in that they contain their own genome which is present alongside the nuclear genome in the majority of eukaryotic cells. Human mtDNA is a small double stranded molecule, 16.5kb in length and encoding 37 genes (22tRNA, 2rRNA and 13 polypeptides) all of which are essential for normal mitochondrial function (Fig1.4).

Mitochondrial DNA is extremely compact. Mutation rates have been calculated as 5 to 10 times higher than its nuclear counterpart. This in turn explains the high degree of polymorphic mtDNA variants which are observed between individuals, the majority of which are neutral (Brown et al, 1979; 1982; Cao et al, 2009). However occasionally mutations arise which are of a pathogenic nature and have the potential to cause disease if present in a great enough number of mtDNA molecules. The first mutations were identified in 1988 (Holt et al, 1988; Wallace et al, 1988) and initially thought to be quite rare. Since then, the incidence of known pathogenic mutations amongst the general population has increased significantly with at least 1 in 10,000 of the adult population affected by mtDNA disease (Schaefer et al, 2008; Turnbull et al, 2009). Hence mitochondrial DNA mutations are increasingly becoming recognised as important causes of human disease.

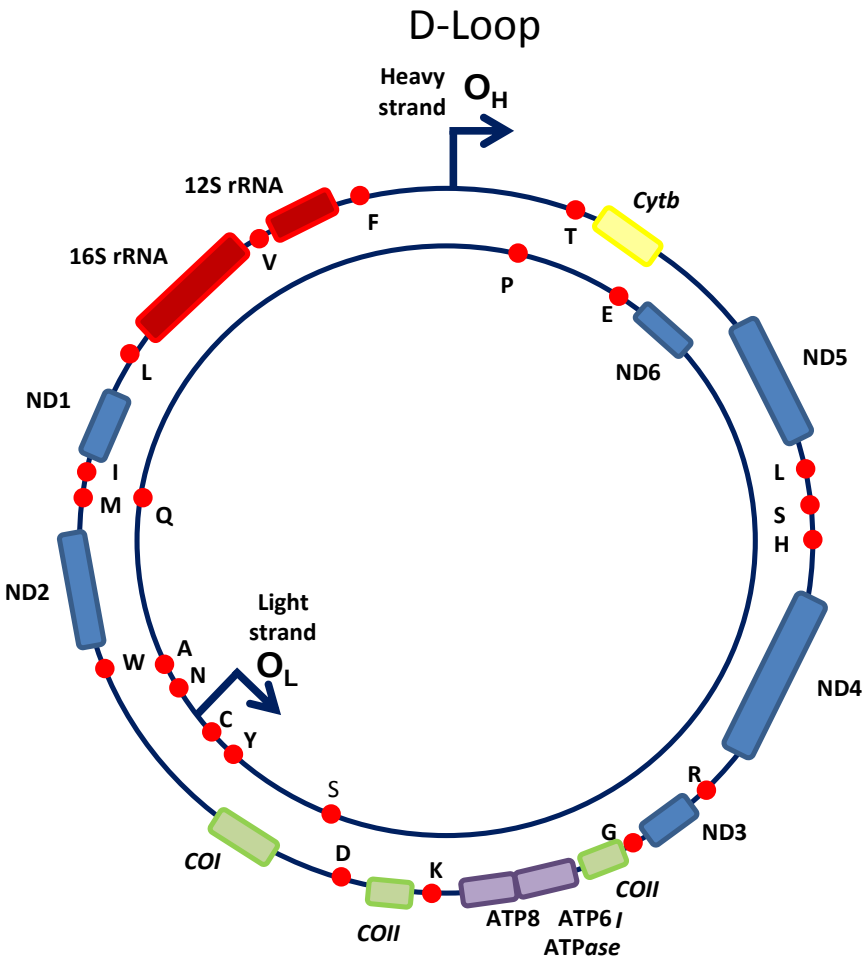


Figure1. 4 The Mitochondrial genome

Map depicting the, 16.5Kb mitochondrial genome. The mitochondrial genome encodes 22 mt-tRNAs (red circles), 2 mt-rRNA genes (red boxes) and 13 protein coding genes (these encode proteins involved in respiratory chain complex assembly). Here the single- letter code is given to represent each of the 22 tRNA genes encoded by the mitochondrial genome. (COI, Complex I; COII, Complex II; COIII, Complex III; ND1, NADH Dehydrogenase, subunit 1 ; ND2, NADH Dehydrogenase, subunit 2 ; ND3, NADH Dehydrogenase, subunit 3;ND4, NADH dehydrogenase subunit 4; ND5, NADH Dehydrogenase Subunit 5; ND6, NADH Dehydrogenase, subunit 6 (blue boxes)).

1.2.2 Mitochondrial genome organisation

Mitochondrial DNA (mtDNA) was once thought to exist in an unorganised free form within the mitochondrial matrix however it is now clearly apparent that mtDNA is indeed organised into macromolecular assemblies we now refer to as nucleoids (Glikerson, 2009). Nucleoids are believed to be involved in the organization and maintenance of mtDNA and are approximately 70nm in diameter (Iborra et al, 2007). The discrete nature of the mitochondrial nucleoid provides an efficient packaging of mtDNA for maintenance and propagation (Gilkerson, 2009).

1.2.3 MtDNA Replication

Currently there are two proposed models of mtDNA replication. The initial model of replication termed 'strand displacement' or the 'asynchronous model' and the second referred to as the 'strand-coupled model' or 'symmetric model'

1.2.3.1 Strand displacement model

The 'strand displacement' model of replication was first proposed by Robbertson *et al* (1972) who by the use of electron microscopy (EM) demonstrated that fully duplex replicative intermediates were not present within the cells under observation (Bogenhagen and Clayton, 2003). However the EM studies did reveal the occurrence of partially replicated displacement loop (D-loop) regions. This was later mapped to a unique location within the mitochondrial genome to only later become referred to as the 'control region' said to be generated by the delayed completion of the newly synthesized L-strand. These initial studies were later confirmed by studies performed by Tapper and Clayton (1981; 1982) and Kang et al (1997).

According to the strand displacement model of replication, synthesis of the leading strand is initiated at the origin of heavy (H)-strand synthesis (O_H) which then proceeds uni-directionally, displacing the parental H-strand as single stranded DNA (ssDNA). The enlargement of the D-loop proceeds for a distance of about two thirds around the circular genome (Brown et al, 2005).

The origin of replication for the lagging strand, termed the origin of light (L) - strand synthesis (O_L), is then exposed, and synthesis of the nascent L-strand proceeds in the opposite direction on the single stranded template of the expanded D-loop (Exp-D).

With the asymmetric model of mtDNA replication one of the resulting daughter molecules with its new L-strand is delayed in completion with respect to the other daughter molecule and therefore segregates prior to fully completing replication. This leaves an area of the H-strand region single stranded which is termed 'gapped circles' since they contain a gap in the newly synthesized L-strand DNA. However there are those who dispute the 'strand displacement' model of replication on the basis that the model is based upon an artefact of preparation in which discontinuously synthesized 'lagging' strands are preferentially degraded due to both a very high rate of ribosubstitution in that strand and the high RNAse H –like activity which is present as contaminant in the preparations (Brown et al, 2005) (Fig1.5a).

1.2.3.2 Strand coupled model

However several papers re-examined the proposed 'strand displacement model' of mammalian mtDNA replication and proposed a new model, referred to as the 'strand coupled' model of mtDNA replication (symmetric replication) (Holt et al, 2000; Yang et al, 2002; Bowmaker et al, 2003; Yasukawa et al. 2005) using a technique based on the 'Brewer and Fangman 2D-gel electrophoresis method' (Friedman and Brewer 1995).

Following these methods, Holt and coworkers (Holt et al. 2000) revealed y-arcs in restriction digest-generated fragments corresponding to an area between the two origins of replication. Replication forks of this type in this mtDNA region are inconsistent with the asymmetric strand-displacement model of replication (Holt et al, 2000). Holt, Jacobs, and collaborators (Yang et al, 2002; Bowmaker et al, 2003) therefore concluded that mtDNA replicates symmetrically, with leading- and lagging- strand synthesis progressing from multiple, bidirectional replication forks within a broad zone (see Fig.1.5b). They also proposed that the strand-displacement model of replication is based on an artifact of preparation in which discontinuously synthesized "lagging" strands are preferentially degraded due to both a very high rate of ribosubstitution in

that strand and an RNase H-like activity that is envisioned as a contaminant in those preparations (Holt et al, 2000; Brown et al, 2005) (Fig1.5b).

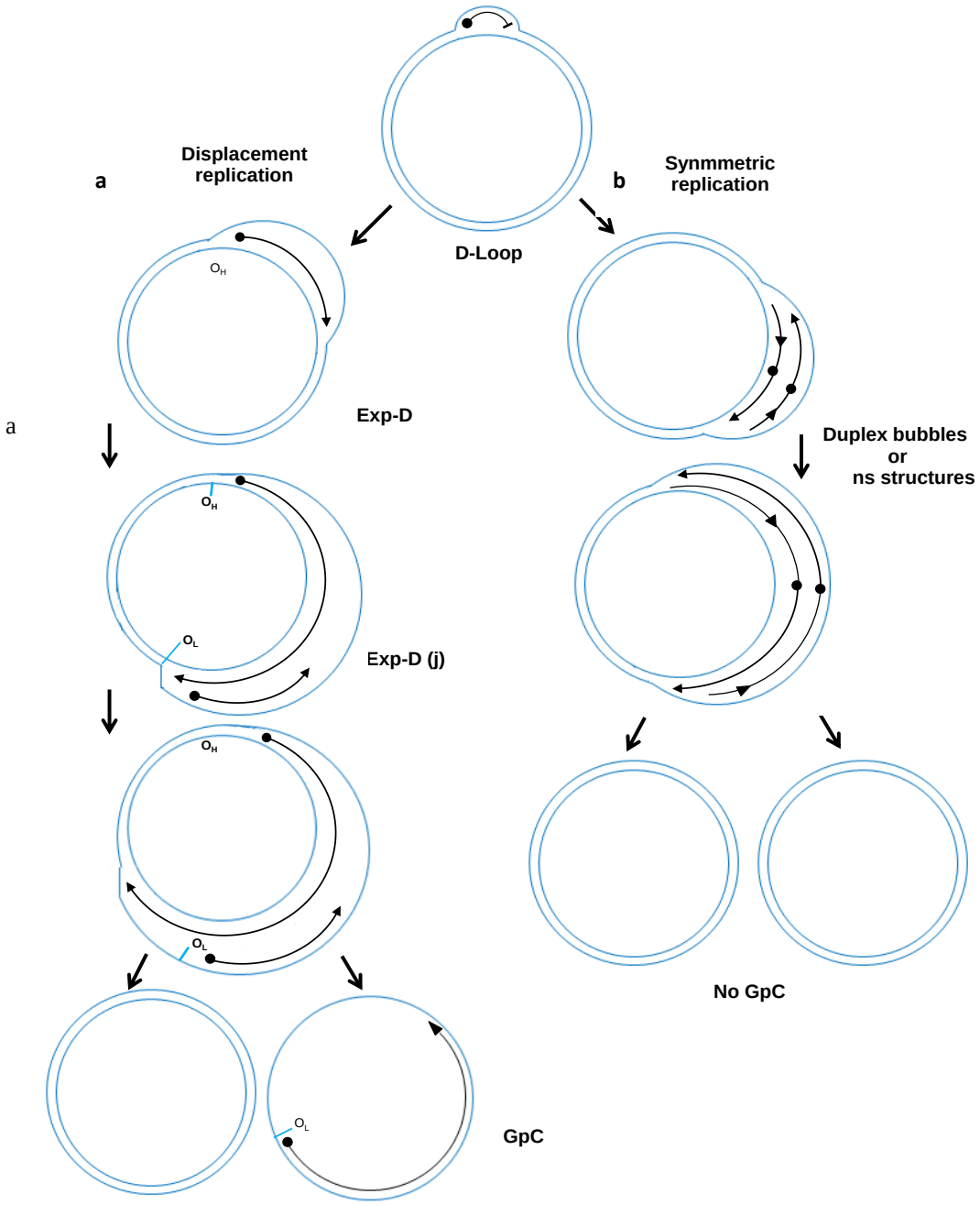


Figure1. 5Comparing asynchronous (displacement) model of replication to conventional strand-synchronous (symmetric) replication.

(a) The displacement model of replication proceeds with single-stranded replication of the H-strand with further expansion and displacement of the D-loop. The intermediates are called expanded D-loops (Exp-D). The Expanded D-loop intermediates continue until the L-strand origin (O_L) becomes exposed, at which point synthesis of the new L-strand commences in the opposite direction. This mode of replication ultimately results in the production of complete daughter molecule and one incompletely synthesized L-strand, called a gap circle (GpC). (b) The Strand coupled model of replication (a-synchronous) assumes there to be a zone of replication intuition, which encompasses an area beyond the simple D-loop. It is from within this zone that both strands are synthesized bi-directionally as the double-stranded replication forks proceeds through the length of the mtDNA.

1.3 Mitochondrial DNA Inheritance

1.3.1 Maternal inheritance

Mitochondrial DNA is inherited through the maternal lineage inclusively with all paternal mitochondria contributed at the point of fertilisation targeted for degradation by ubiquitin soon after their introduction into the ooplasm of the mature ovum (see below).

As all mitochondria in the zygote derive from the ovum a mother harbouring an mtDNA mutation could potentially transmit this to future children to some varying degree, however only her daughters will pass it on to their progeny. The degree to which her offspring are affected can vary vastly due to a phenomenon known as the 'bottleneck effect' which filters and ultimately determines which mitochondria each offspring will receive.

1.3.1 Destruction of paternal mitochondria

It has been estimated that human spermatozoa contain between 700 and 1200 copies of mtDNA (Diez-Sanchez et al, 2003) which enter the oocyte at fertilisation and are present within the cytoplasm. However as mentioned previously the paternal mitochondria are eliminated from the oocyte, shortly after fertilization (Fig 1.6). The exact developmental stage at which this occurs and the precise mechanism by which the paternal mitochondria are removed varies between published studies. Currently there are two mechanisms which have been proposed to explain the removal of paternal mitochondria. The first invokes the ubiquitin tagging system in the selective removal of sperm mitochondria inside the fertilised egg (Sutovsky et al, 1999). The second hypothesis proposes that upon fertilisation several sperm components including mitochondria, enter the oocyte and are rapidly degraded by autophagy (Rawi et al, 2011).

However there is some evidence which suggests that on rare occasions paternal mitochondria are able to evade elimination and contribute to the population of mitochondria which go on to populate the embryo (Bromham et al, 2003). An example of this extremely unusual paternal transmission is provided by a single report of a

pathogenic mtDNA mutation within paternally derived mtDNA in a patient with muscle myopathy (Schwartz and Vissing, 2002).

Introduction of IVF techniques which involve the direct injection of sperm into the oocyte has been suggested as an artificial route of entry which could facilitate the contribution of paternal mitochondria to the zygote (Sutovsky et al, 2004; Spikings et al, 2006). However there is insufficient evidence to support this, but there is strong evidence to suggest the contrary (Houshmand et al, 1997; Danan et al, 1999; Marchington et al, 2002).

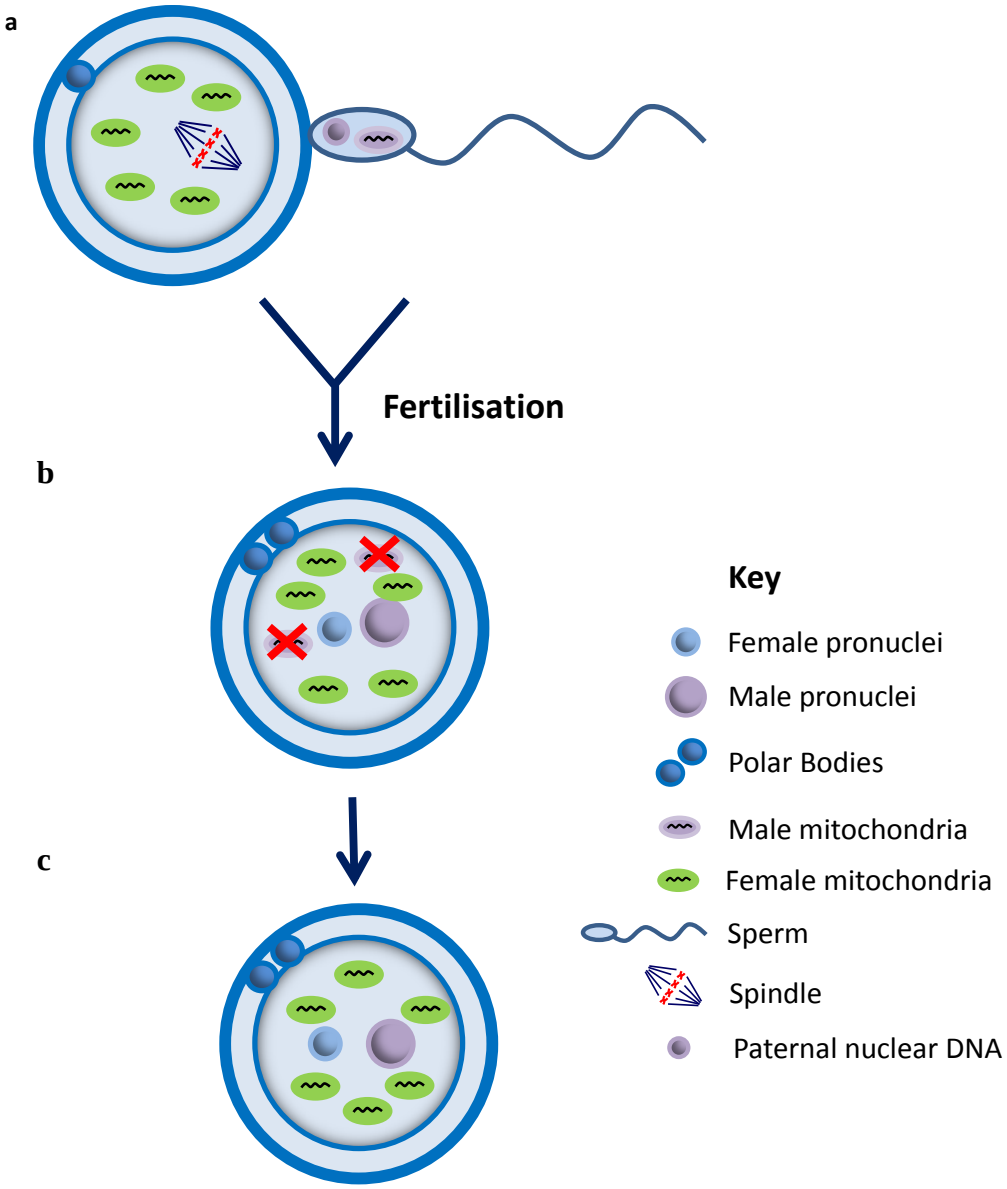


Figure1. 6 Destruction of paternal mitochondria

This schematic diagram depicts the removal of paternal mitochondria upon fertilization, resulting in the strict maternal inheritance of all mtDNA. (a) The mature oocyte contains many thousands of mitochondria (shown in green) whilst the sperm contains relatively few mitochondria (shown in purple). (b) Shoetly following fertilisation, paternal mitochondria is introduced into the oocyte, are targeted for destruction. (c) The resulting embryo contains only maternally-inherited mitochondria.

1.3.2 Mitochondrial DNA mutations

Since the identification of the first mitochondrial pathogenic mutation in 1988, >300 have been documented. These take the form of point mutations or rearrangements (deletions or duplications). These either affect protein synthesis if they alter a tRNA or rRNA or alter the normal functioning of the respiratory chain if they lie within one of the genes which encode one of the subunits or enzymes of the respiratory chain (Turnbull et al, 2009).

1.3.2.1 MtDNA point mutations

Numerous mtDNA point mutations have been documented in protein encoding genes, tRNAs and rRNAs. However the criteria for defining pathogenic point mutations from mere harmless polymorphic mutations has proven difficult due to the complex nature which exists between the lack of genotype and phenotype correlation for some mitochondrial diseases (DiMauro and Schon, 2001; Schapira, 2006). Examples of mitochondrial DNA diseases associated with point mutations within protein coding genes include maternally inherited Leigh syndrome (MILS) caused by a point mutation in the *ATP6* gene (m.8993T>C), and Leber's hereditary optic neuropathy (LOHN) which is usually due to one of three mutations all found within the *MTND* gene of complex I (m.3460G>A, m.11778G>A and m.14484T>C). Point mutations within the mitochondrial tRNA genes may affect the function of oxidative phosphorylation by disrupting both the structure and function of the mt-tRNA molecule, resulting in impaired mitochondrial translation. Examples of mitochondrial DNA diseases associated with point mutations within tRNA genes include mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) due to a point mutation within the *MT-TL1* gene (m.3243A>G), and myoclonic epilepsy and ragged red fibres (MERRF) which is commonly caused by a point mutation in the *MT-TK* gene (m.8344A>G).

1.3.2.2 MtDNA deletions

Mitochondrial deletions are a primary cause of mtDNA disease, which can occur as single or multiple mtDNA deletions. Single mtDNA deletions are a common cause of sporadic mitochondrial disease, with the identical mutation present within every cell of the individual (Schaefer *et al*, 2008). Single mtDNA deletions usually occur

sporadically in early development, although there are several reports of mtDNA deletions that appear to have been maternally transmitted (Bernes et al, 1993; Shanske et al, 2002). Examples of mitochondrial DNA diseases associated with single large-scale deletions include Kearns-Sayre syndrome (KSS) and chronic progressive external opthalmoplegia (CPEO), which both involve deletion of several mitochondrial genes. However there are also patients who harbour multiple mtDNA deletions, which are primarily thought to arise due a defect in a nuclear encoded gene. The proteins which are affected are normally involved in mitochondrial nucleotide metabolism or mtDNA maintenance. Multiple mtDNA deletions are usually found at higher levels in the muscle and central nervous system (Taylor and Turnbull, 2005; Krishnan *et al*, 2008). There are also numerous reports which link mtDNA deletions with neurodegenerative disease (Beal, 2004; Reeve et al, 2008; Gu et al, 2002).

The exact mechanism by which mtDNA deletions occur has yet to be fully resolved. Currently there are two primary schools of thought as to how deletions arise the first during replication of mtDNA and the second during repair of damaged mtDNA (Krishnan et al, 2008). Currently the slipped -strand mode of replication is favoured by most scientists. However there are flaws to this suggested mechanism or this to workthe mtDNA would not replicati and furthermore replicating at the point where the single stranded 5 and 3 repeats come together to form a loop when the strand break occurs (krishnan et al, 2008). Krishnan et al (2008) was the first to propose formation of mtDNA deletions through repair of damaged mtDNA. This model proposed that mtDNA deletions are initiated by single-stranded regions of mtDNA generated through exonuclease activity at double stranded breaks. The single strands generated would then be free to anneal with the repeat sequences present single stranded.

1.3.3 Homoplasmy and Heteroplasmy

There are thousands of mtDNA molecules per cell, with copy number varying depending upon cell type and function. Usually a cell will contain identical copies of the wild-type mtDNA sequence. However where there is an mtDNA mutation which arises in the germ line, all copies present in the cell may then contain this mutation, which would be referred to as a homoplasmic mutation. Equally only a proportion of mtDNA may possess the mutation and this is referred to as heteroplasmy (Turnbull et al, 2009) (Fig1.7).

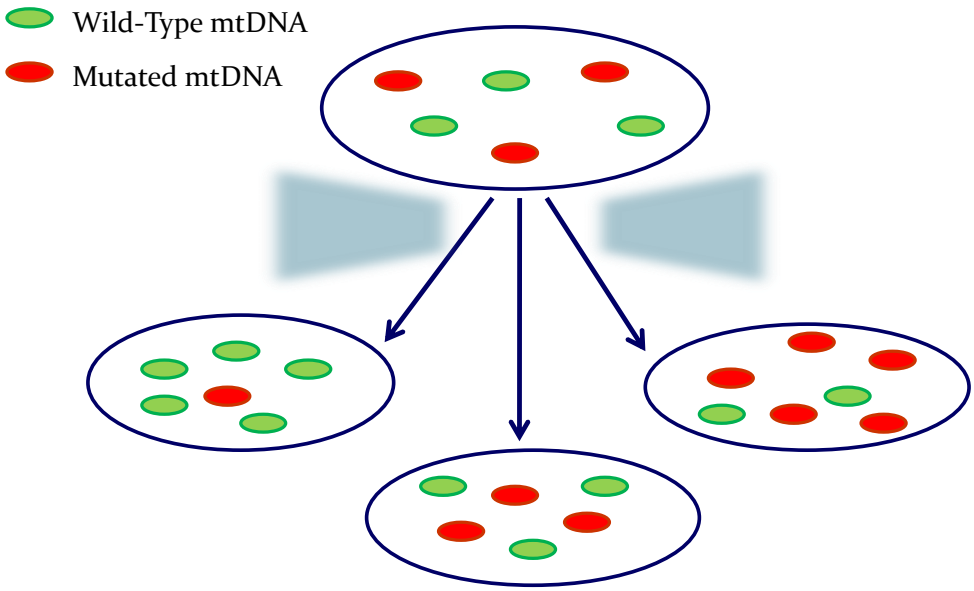


Figure1. 7 Heteroplasmy and Homoplasmy

Schematic diagram representing the three different possible situations which may be observed for mitochondrial distribution. If a cell contains copies which are all affected a cell would be described as 'homoplasmic' for this particular mutation, if an individual cell receives a mixture of affected and normal mitochondria this would be said to be 'heteroplasmic' and finally a cell which has a full complement of normal unaffected mitochondria, as wild-type.

1.3.4 Threshold Effect

The ratio of mutant to wild type mtDNA plays a pivotal role in determining the degree to which an individual is affected by mitochondrial disease. Typically between 60-90% mutated mtDNA is required for a clinical phenotype to develop which is usually referred to as the 'threshold' effect. However this margin can vary depending upon the specific mtDNA mutation and the cell type under examination (Chinnery et al, 1997).

Furthermore, in a proportion of individuals who present with a heteroplasmic mtDNA mutation, not all cells will show evidence of a biochemical defect, which can be the result of a tissue-specific pattern of segregation, where tissues which exert high energy demands exhibit high mutation loads (Turnbull et al, 2009) (Fig1.8).

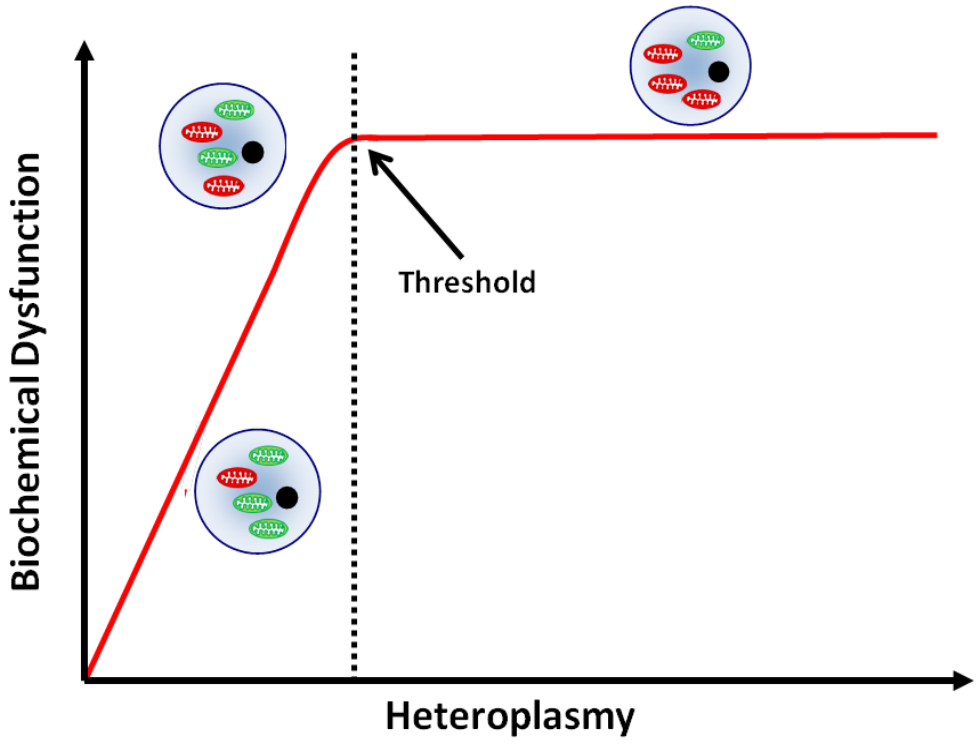


Figure1. 8 The threshold effect

Under normal conditions healthy individuals will contain one type of mtDNA molecule referred to as wild type mtDNA. However wild-type and mutant copies of mtDNA can co-exist within the same cell. When this occurs a state of heteroplasmy is said to exist. A third state can also exist, when all copies of mtDNA are mutated which is referred to as homoplasmy. The ratio of wild-type to mutated mtDNA contained within an individual cell can vary between cells and often tissues within the same individual carrying a, mtDNA mutation. The point at which a biochemical defect is detected within an individual, using molecular techniques is known as the 'threshold'. The threshold often differs and is highly mutation dependent.

1.3.5 The mtDNA Bottleneck

Females carrying a mixture of mutant and wild-type mtDNA variants run the risk of transmitting a variable amount of mutant mtDNA to each offspring (Cao *et al*, 2009). The exact mechanism which governs how and which mitochondria are transmitted to future offspring is still not clear. However the presence of 'genetic bottleneck' during early embryonic development is believed to be responsible for the variable levels of mutated mtDNA observed in affected offspring from mothers with pathogenic mtDNA mutations (Taylor et al, 2005).

Therefore although a 'genetic bottleneck' is known to be responsible for creating the variation we observe, the exact point during embryonic development at which this genetic bottleneck occurs is still unknown. However there are several studies which have attempted to unravel the bottleneck in order to better understand why mtDNA inheritance is prone to such variance. There are several different mechanisms which could explain the differing levels of heteroplasmy we observe within families affected by mitochondrial disease. This includes; a marked reduction in the number of mtDNA molecules being transmitted from mother to offspring (the mitochondrial genetic bottleneck), the partitioning of mtDNA into homoplasmic segregating units, the selection of a group of mtDNA molecules to re-populate the next generation (Cree et al, 2008). Three different studies have been performed to try and shed further light on the genetic bottleneck, each reaching different outcomes, which are described in greater detail below.

The first model proposed by Cree et al (2007), suggests that the bottleneck results from a 'physical' process combined with a dramatic reduction in mtDNA copies during pre-implantation and post-implantation development. Together this is said to create the genetic variation in heteroplasmy levels seen among offspring of heteroplasmic mtDNA mutation carriers. The majority of the heteroplasmy variance (~70%) they were able to show experimentally would be created as a result of physical partitioning of the mtDNA molecules into daughter cells during pre- and early post-implantation development when the amount of mtDNA in each cell is relatively low (~200 copies). The remainder of the variation (~30%) is likely to occur during the intense proliferation of mtDNA in the exponentially expanding primordial germ cell (PGC) population (cells which give rise to the gametes), where the average amount of mtDNA was ~1,500

molecules per PGC. This study also disputed the theory that the mtDNA genetic bottleneck was the result of compartmentalization of the mtDNA molecules into multi-copy segregating units such as nucleoids or through the selection of a particular group of mtDNAs chosen to repopulate the next generation.

However the second model proposed by Cao et al (2009) concluded that the genetic bottleneck is generated without a dramatic reduction in mtDNA content in germ cells (PGCs). They also postulated that the genetic bottleneck is likely to result from the replication of a small effective number of segregating units each containing multiple copies of mitochondrial genomes during oocyte maturation. This they said was likely to occur in combination with a strong purifying selection against severe mutations over a long period of time in the germ line (Cao et al, 2009).

The third mechanisms proposed by Wai et al (2008) also suggests, that the genetic bottleneck is likely to result from replication of only a subgroup of mitochondria. As they're experiments found that only a small sub-group of mitochondria actually appeared to be actively undergoing replication in the primordial and primary follicles. From this data they therefor deduced that there were likely to be two genetic bottlenecks acting at different times during embryonic development. Here a physical bottleneck (~200 mtDNAs) occurring in the earliest PGCs, most likely enables selection against severely deleterious mtDNA mutations. Whilst the second genetic bottleneck which would be responsible for the segregation of neutral sequence variants which they regard to be associated with human disease, occurring instead during folliculogenesis. This would most likely take place in early post-natal life, as a result of replication of a subpopulation of mtDNAs. This can be explained by the high copy number in mature oocytes (~175,000, likely organized as 1-2 copies per organelle) which can be viewed as a genetic device to ensure even distribution of mitochondria to the cells of the early embryo at a time when mitochondrial biogenesis is arrested (Fig1.9).

Understanding what how and why mitochondria are transmitted will ultimately allow us to greatly improve our understanding of mitochondrial inheritance. This information regarding mtDNA content during early embryonic development will facilitate the development of therapeutic strategies blocking the mitochondrial disease transmission from mother to progeny. However as we still do not as yet know why certain mutant mtDNA appear to segregate in a tissue specific manner and why some mutant mtDNA

are lost within several generations while others persist long enough to be transmitted to future generations the question remains unresolved (Schapira, 2006; Chinnery et al, 1998).

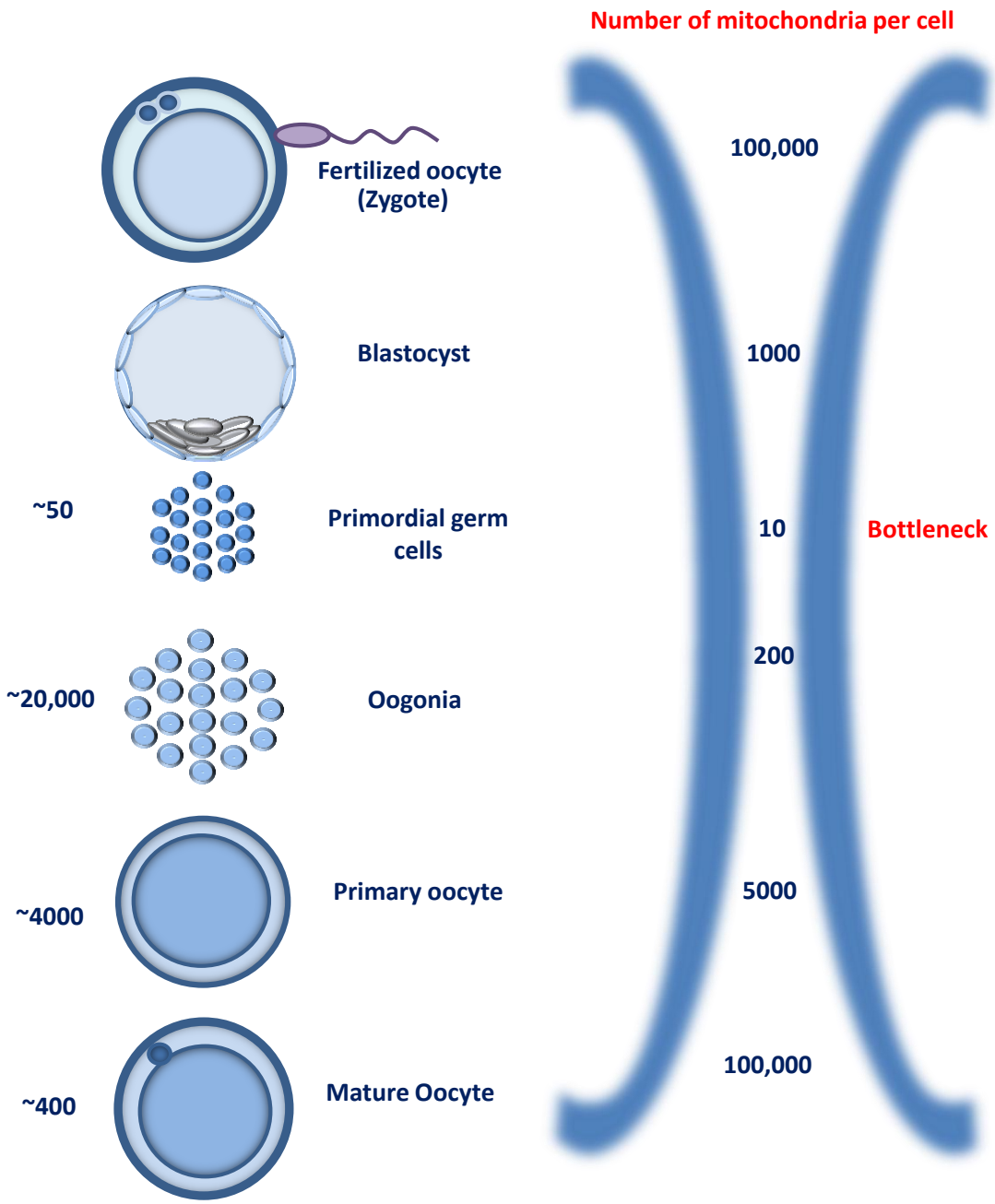


Figure1. 9 The mtDNA bottleneck

Schematic diagram representing changes in the number of mitochondria during development of the female germ line. An estimate of the number of mitochondria present at each stage of germ line development is given (estimates based on approximate numbers present in the mouse). This diagram represents the potential presence of a genetic bottleneck influencing the transmission pattern of mtDNA during the creation of the primordial germ cells. This model also suggests that the segregation of mtDNA sequence variants is essentially complete by the time the primary oocytes are differentiated in foetal life (Adapted from original image by Shoubridge and Wai, 2007).

1.4 Management and diagnosis of mtDNA disease

1.4.1 Diagnosing mtDNA disease

Mitochondrial disease can present with a wide variety of symptoms and is often multisystematic, or affecting specific tissues or organs dependent upon the causative defect (McFarland and Turnbull, 2009; Taylor and Turnbull, 2005). Patients with mtDNA disease have been said to fall in to one of three groups; those with a clearly recognised syndrome which is associated with specific mtDNA abnormality, secondly those who are said to have a cluster of clinical features which suggests that they have a mtDNA defect and finally those who are referred with an unusual clinical presentation which may be the result of a pathological mtDNA mutation (Chinnery and Turnbull, 1997).

There are several common phenotypic symptoms characteristically associated with mtDNA disease: asymmetric ptosis, short stature, migraines/headaches, seizures, neuropathy, diabetes, deafness and proximal limb muscle weakness (Chinnery et al, 1997; MacFarland and Turnbull, 2009). A number of 'classical' syndromes have been described that are often, but not always, associated with a particular genotype (McFarland and Turnbull, 2009). However the association of phenotype with genotype is far from straightforward in mitochondrial disease, often with mutations in mtDNA and nDNA resulting in similar phenotypes (McFarland and Turnbull, 2009). Indeed there is often diverse variety in clinical phenotypes observed between patients known to harbour the same mutation, with the m.3243A>G mutation demonstrating this best, with at least three different phenotypes associated with this single mutation, including; mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), maternally inherited diabetes and deafness (MIDD) and chronic progressive externalopthalmoplegia (CPEO) (McFarland and Turnbull, 2009). In childhood presentations of mtDNA disease, symptoms often tend to be more severe than those with onset in adult-life and can often affect life expectancy (MacFarland and Turnbull, 2009). Mitochondrial disease can present at any age (Moraes et al, 1989).

Most patients with mtDNA disease have either a deletion or point mutation (Moraes *et al*, 1993; Chinnery et al, 1997; Chinnery and Turnbull, 1997). MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes), is

caused by a point mutation in the mitochondrial leucine (UUR) tRNA (MTTL1) gene m.3243A>G. A set of diagnostic criteria was originally proposed for MELAS, including; stroke before the age of 40; an encephalopathy characterized by seizures, dementia or both and finally a blood lactic acidosis or ragged red fibres in skeletal muscle. Both, however, can be present simultaneously (Hirano et al, 1992; Goto et al, 1990; Chinnery and Turnbull, 1997). Another commonly diagnosed mitochondrial disorder is MERRF (myoclonic epilepsy with ragged red fibres) and results from a point mutation in the mitochondrial lysine tRNA gene m.8344A>G (Shoffner et al, 1990). Although there is considerable overlap in clinical presentation of MELAS and MERRF, they are both distinct syndromes resulting from point mutations in two different tRNAs (Chinnery et al, 1997). NARP (neurogenic weakness, ataxia and retinitis pigmentosa), (Holt et al, 1990; Shoffner et al, 1992; Chinnery et al, 1997), is sub-acute encephalopathy in childhood with prominent brain stem signs (Leigh syndromes) (Shoffner et al, 1992). This mutation is most commonly associated with two point mutations mt.8993T>G and mt.8993T>C both located within the MT-ATP6 gene. The mt.8993T>G is generally associated with a more severe clinical phenotype in comparison to the mt.8993T>C variant, which even at similar levels of mutation is generally associated with a much milder and less severe phenotype. Individuals who present with clinical phenotypes reminiscent of NARP syndrome most frequently carry the mutation at high levels; >80% and >75% for the mt.8993T>C and mt.8993T>G mutations, respectively (Steffann et al, 2007). Due to these extremely high mutation levels the risk of recurrence for this particular mutation is often equally as high.

Numerous clinical investigations are often conducted when making a clinically confirmed diagnosis of mitochondrial disease. These include urinary dipstick assessment for blood, protein and glucose to assess potential renal involvement. Blood tests can provide additional supportive evidence, ensuring analysis of creatine kinase, resting blood lactate, full blood count, urea and electrolytes, liver function tests, blood glucose and thyroxine status. Respirometry, electrocardiogram (ECG), lumbar puncture, electroencephalogram (EEG) and MRI can also all be included to aid in making a full clinical diagnosis (MacFarland and Turnbull, 2009).

Muscle biopsies are often performed to allow for histological and histochemical analysis, which still remains viewed as the ‘gold standard’ for the detection of mitochondrial disease (Taylor et al, 2004). A battery of histochemical assays are, now

routinely performed on muscle biopsy tissue, including the Gomori trichrome stain, succinate dehydrogenase (SDH) assay, and cytochrome *c* oxidase (COX). The additional information provided by these mitochondrial enzyme histochemistry assays is often combined and interpreted alongside clinical, personal information and results of biochemical respiratory chain analysis. This provides a comprehensive diagnosis. Increasingly, molecular genetic analysis is being employed in the investigation of mitochondrial disease. This is often following clinical, histochemical and biochemical analysis as this allows for a rational approach to molecular investigations to be undertaken.

1.4.2 Management of mitochondrial disease

Following a confirmed diagnosis of mitochondrial disease in patients, management of the condition is the best that can be hoped due to the current lack of therapies available at present to alter disease course (Chinnery *et al*, 1997).

1.4.2.1 Pharmacological treatments

Pharmacological treatments have often been considered to be of limited benefit to mtDNA patients providing only short lived symptomatic improvements (McFarland and Turnbull, 2009). This is partly due to the multisystematic nature of mitochondrial disorders which means many different organ systems are affected. Indeed those organs and tissues which are often worse affected by mitochondrial dysfunction are those which have the highest energy demands and rely upon mitochondria to function correctly. This includes organs such as the heart, brain, liver and kidneys and tissues such as muscle.

As a consequence patients who present with mtDNA disease often present with a broad spectrum of symptoms, which are often unique to that patient alone. Indeed even patients who are known to harbour the same pathogenic mutation can be present with a different set of symptoms. This has in the past made it incredibly difficult to tailor pharmacological treatments to suit all patients, as just as one treatment may help provide relief from symptoms for one patient it may not be able to do so for other patients due to the nature of mtDNA disease.

Other issues which have hampered the success of studies examining pharmacological interventions, includes the relative small number of patients able to be included in trials (Taylor et al, 1997). This is a result of the wide range of mtDNA mutations which can cause mtDNA disease, the relatively small number of patients which in-turn have been diagnosed with each known mutation, the diverse range of symptoms patients can present with, the effect of heteroplasmy and also effects of nuclear genome interactions. Meaning that the numbers of patients able to be included in specific drug trials is relatively low. As a result it makes it difficult to assess the effectiveness and safety of pharmacological treatments.

However mitochondrial medicine is a unique discipline that is evolving owing to advances in technology and knowledge regarding the role of mitochondria in disease development. Furthermore the unique structural and functional characteristics of mitochondria allow for selective intracellular targeting of drugs designed to modulate mitochondrial function for therapeutic gain. This is because mitochondria play a key role in; the regulation of energy metabolism, reactive oxygen species (ROS) production and apoptosis. Therefore, the specific delivery of drugs to mitochondria may provide the foundation to treat a variety of diseases wherein these functions are deregulated (Szewczyk and Wojtczak, 2002; Armstrong, 2007).

Some of the potential therapeutic applications which have been proposed by utilising mitochondrial targeting include; the delivery of antioxidants to mitochondria to prevent oxidative damage associated with the neurodegenerative diseases, ischemia, tissue injury and diabetes. Secondly the targeting of toxic drugs or Bcl-2 proteins to the mitochondria to trigger apoptosis in cancer therapy. Thirdly the delivery of drugs to mitochondria to inhibit the mitochondrial permeability transition (MPT) which occur following heart attacks and stroke. And fourthly the targeting of drugs to either uncouple the electron transport chain (ETC), or activate the uncoupling proteins which are involved in obesity and diabetes. However, the development and implementation of successful therapeutic treatments targeted at disorders resulting from mitochondrial dysfunction are still some way off. This is due to both a lack of knowledge of the potential long-term toxic effects associated with some of the compounds being investigated for their use and secondly a current lack of efficient methods to regulate drug delivery to the tissue of interest (Armstrong, 2007).

1.4.2.2 Dietary supplementation

Supplementation of diet with vitamins and minerals has shown to help in the management and alleviation of symptoms in patients with mitochondrial disease. Here vitamins C and E, both anti-oxidants, are routinely prescribed to patients with mitochondrial disease although their role in ameliorating symptoms is often minor or absent (Sheu et al, 2006). Dietary supplementation with Ubiquinone (coenzyme Q10) has also shown promise (Matthews et al, 1993; Gold et al, 1996). Indeed a study published in 2010 (Dai et al, 2011) reported observing a reversal of mitochondrial dysfunction following coenzyme Q10 supplementation, however improvements were limited to improved endothelial function in patients with left ventricular systolic dysfunction (LVSD).

1.4.2.3 Exercise studies

Exercise intolerance in patients with heteroplasmic mtDNA mutations is predominately due to high ratios of mutant to wild-type mtDNA, which subsequently impairs the capacity to increase oxidative energy production during times of increased demand (Murphy et al, 2008). Aerobic training has been suggested as a means by which to improve muscle oxidative capacity and compensate for the mtDNA defect (Taivassalo et al, 1998; Taivassalo and Haller, 2005; Murphy et al, 2008). Studies have demonstrated that fatigue and tolerance to daily activities does improve as a result of exercise training. However the concept of exercise training as a therapy for mitochondrial disease patients has been questioned due to concerns over general safety and efficacy (Taivassalo and Haller, 2005; Taivassalo et al, 2006). Resistance training has also been examined, in order to help improve and increase muscle strength (Murphy et al, 2008).

1.4.2.4 Personalised gene therapies

Due to the lack of effective treatments currently available for patients with mtDNA disease, the prospect of selective inhibition of mutant mtDNA replication has in recent years been explored (Taylor et al, 1997). Peptide nucleic acids (PNA's) have been suggested as one potential means by which this could be achieved. It was hoped that PNA's could be employed to selectively inhibit the replication of the mutant mtDNA molecules and theoretically allow replication and propagation of only the wild-type

mtDNA. However many issues concerning this approach still have as yet to be overcome, including the uptake of PNA's into cells and the stability of the PNA's once bound to the mutated copies of mtDNA.

1.5 Female Gametogenesis and Early Preimplantation Development

1.5.1 Oogenesis

Oogenesis is the female process of gametogenesis ultimately leading to the formation of an ovum (egg cell). This is initiated when the primordial germ cells migrate to the genital ridge, where they then differentiate to form oogonia (Sadler, 2009). Oogonia then undergo a number of mitotic divisions, where upon they form clusters, which become surrounded by a layer of flat epithelial cells, otherwise known as follicular cells. Some then arrest their cell division in prophase I and form primary oocytes. By the fifth month of prenatal development (~20 weeks) oogonia reach their maximum number of about 7 million which is vastly reduced by birth to ~1-2 million in a process termed atresia. All those surviving primary oocytes have entered prophase of meiosis I. Near the time of birth, these primary oocytes (primordial follicles) have arrested at the diplotene stage, which is characterized by a lacy network of chromatin. They remain arrested at this stage of prophase until the onset of puberty (Sadler, 2009).

During the first meiosis the primary oocyte becomes the secondary oocyte and extrudes its first polar body. Following the extrusion of its first polar body the secondary oocyte proceeds into meiosis II. This is halted at the metaphase II stage ready to be fertilised. Upon fertilisation, the secondary oocyte extrudes its second polar body. Both polar bodies should then degenerate, their only function being to discard the extra haploid set of chromosomes created at each cell division of meiosis (Fig1.10).

1.5.2 Mammalian Meiosis

Meiosis is the cell division which takes place in the germ cells to generate male and female gametes. It is a reductional division in which the number of chromosomes per cell is halved, eventually resulting in the production of four haploid gametes. Meiosis therefore requires two cell divisions meiosis I and meiosis II, to reduce the number of chromosomes to the haploid number of 23 (Sadler, 2009).

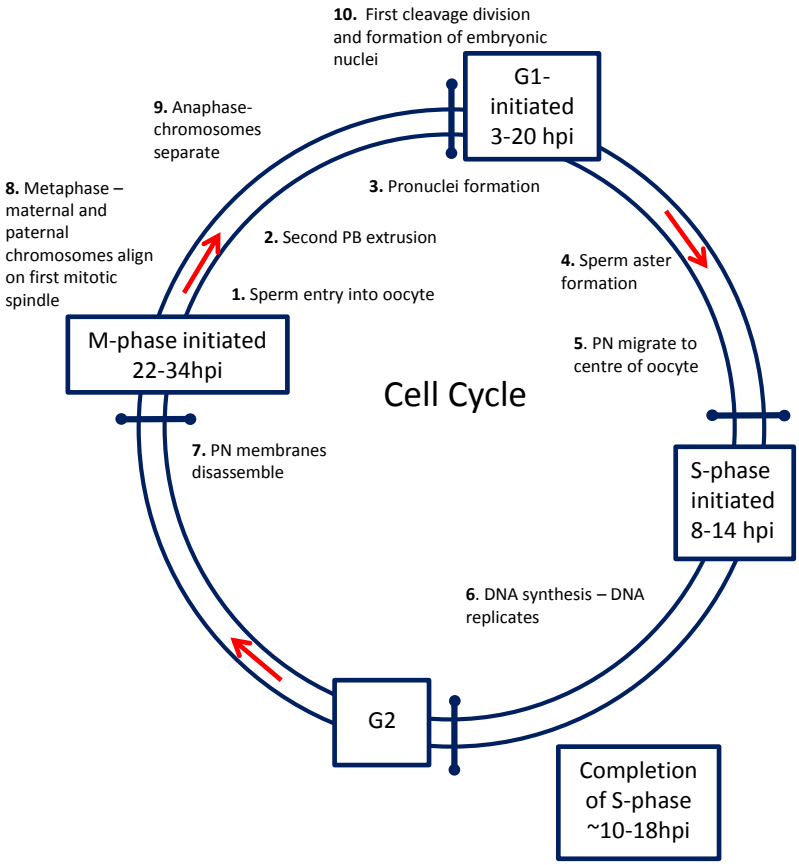


Figure1. 10 Events of fertilization and pronuclear formation in relation to the cell cycle

Schematic diagram to display the key events which take place during fertilisation and pronuclear formation in relation to the cell cycle. The oocyte is arrested in metaphase II of meiosis; sperm entry then triggers resumption of meiosis and the oocyte extrudes the second polar body. The presence of the male and female pronuclei marks the transition of the zygote in to G1 of the mitotic cycle. This has been shown can occur between 3-20 hours post insemination/injection (hpi)(Balakier et al, 1993b; Capmany et al 1996; Payne et al, 1997; Nagy et al, 1998)(Image taken from Feenan and Herbert, 2006). A sperm aster then formed in close proximity to the male pronucleus, and the microtubule arrangement draws the female pronucleus towards the male until the two pronuclei abut in the centre of the oocyte. The cell enters the S-phase (8-14hpi) and DNA synthesis is completed (~10-18hpi) (Balakier et al, 1993b; Capmany et al, 1996). Upon completing S-phase, G2 is initiated, which lasts for around 4-6 hours (Blakier et al, 1993b). At the end of G2, the pronuclear membranes disassemble, the paternally inherited centrosome coordinates mitotic spindle formation, and the male and female chromosomes align o the spindle at the metaphase. M-phase is entered into around 22-31 hpi. Sister chromatids separate at anaphase and each daughter cell inherits a set of maternal and paternal chromosomes.

1.5.3 Fertilisation

The pronuclear stage represents a unique transition stage from meiotic to mitotic cell division. Here sperm has entered the oocyte at which point the oocyte completes its second meiotic division. In normally fertilised eggs two pronuclei exist, a maternal and paternal pronucleus, each containing a haploid number of maternal or paternal chromosomes. The pronuclei generally exist as two separate entities until their membranes breakdown at the onset of the first mitotic division (Plachot, 2000; Feenan and Herbert, 2006) (Fig1.12).

1.5.4 Abnormally fertilised human embryos

In a small proportion of IVF cycles, the selection is limited to embryos that are abnormally fertilised, displaying one or three pronuclei. Embryos are considered abnormal when they have more or less than 2PN. These zygotes therefore contain either 1 or >3PN and can result from an array of scenarios; for example a 1PN embryo can result when the female pronucleus fails to form and a single male pronucleus may only be visible (Feenan and Herbert, 2006). It is also possible however that an oocyte with 1PN and two polar bodies may have been parthenogenetically activated and resumed meiosis independently of a sperm cell (Staessen et al., 1993). This results in formation of a haploid female pronucleus and cytoplasm devoid of any nuclear DNA (Levron et al., 1995). It has been also been reported that a proportion of 1PN embryos are actually diploid (Levron et al., 1995), and that such embryos could be formed by asynchronus pronuclear development (Munne et al., 1993) or pronuclear fusion (Tesarik and Mendoza, 1996).

However the most frequently observed multipronucleate embryos are those containing 3PN (Feenan and Herbert, 2006). The extra pronucleus may be either maternal or paternal in origin and this can be inferred by the number of polar bodies. An embryo with 3PN and two polar bodies is assumed to be dispermic and are formed following entry of two sperm in the oocyte, resulting in a single maternal pronucleus and two paternal pronuclei. An embryo with 3PN and only one polar body may form if the oocyte fails to extrude a polar body during oocyte maturation (meiosis I) or activation

(meiosis II), resulting in formation of two maternal pronuclei and a single paternal pronucleus. This may occur following intracytoplasmic sperm injection if the sperm is introduced in the vicinity of the meiotic spindle (Macas et al, 1996).

1.5.5 Pre-implantation development

Pre-implantation development in mammals involves both the development of the embryo and the preparation of the uterus in anticipation of blastocysts implantation (Stuart and Cullinan, 1997). After the first cleavage divisions, which are under maternal genome control, the segmentation continues under the control of the embryonic genome (Tesařik, 1998). Here the flattening of the blastomeres and the formation of specialised junctions amongst them characterize the foundation of the two different cell populations of the early blastocyst (Tesařik, 1993). These two cell populations will later give rise to the inner cell mass and the trophoectoderm, which are finally segregated during cavitation (Tesařik, 1993) (Fig1.11).

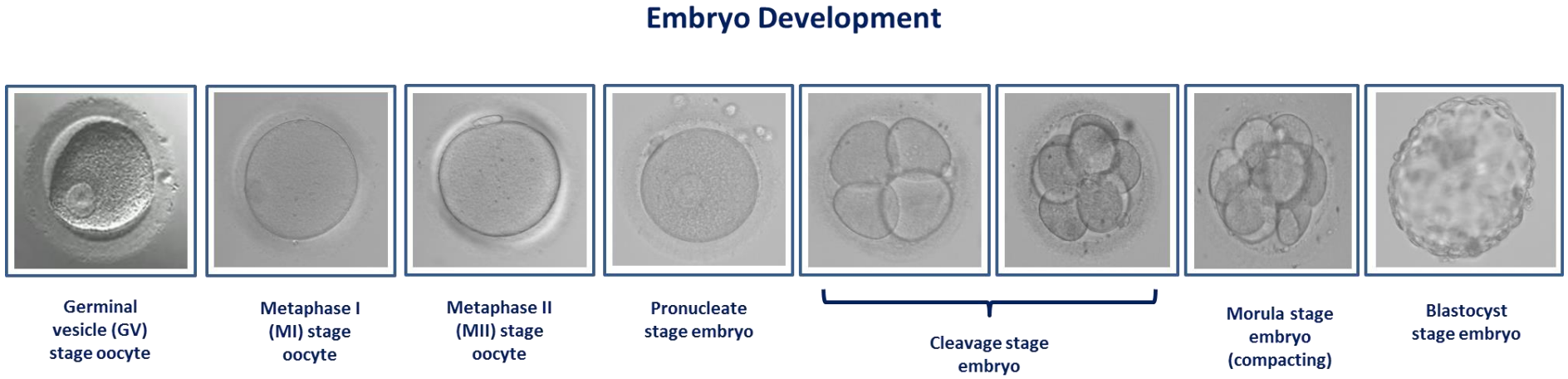


Figure1. 11 Embryo development

Above images represent the various stages of embryo development (a) Germinal vesicle (GV) stage oocyte (b) Metaphase I (MI) stage oocyte (c) Metaphase II (MII) stage oocyte (d) Pronucleate stage (e) 4-cell cleavage stage embryo (f) 8-cell cleavage stage embryo (g)compacting early morula (h) expanded and hatching blastocyst

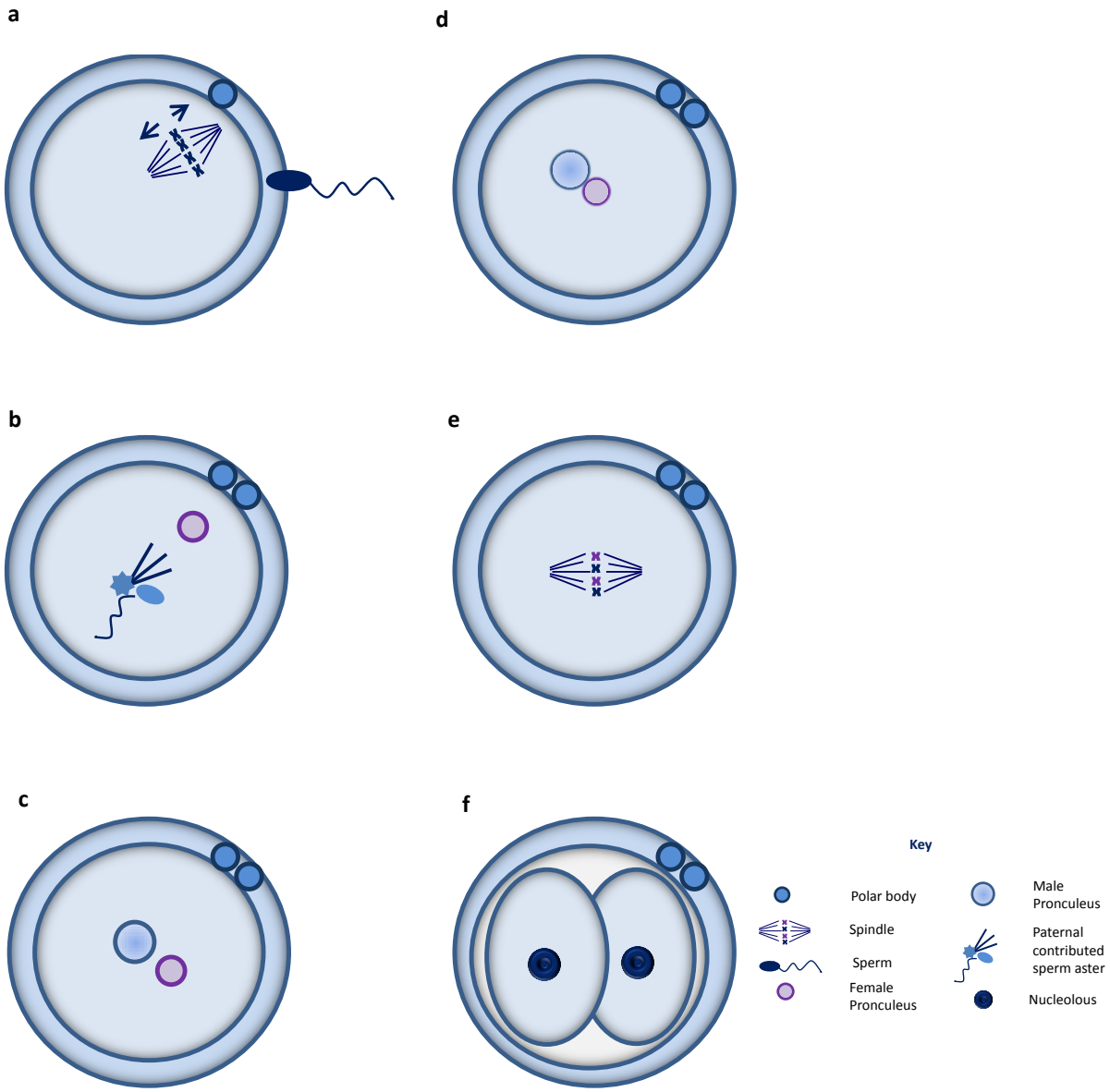


Figure1. 12 Pronucleus formation

Above diagram the series of events which take place following fertilisation to the first cell division. (a) Sperm enters the oocyte, which leads to the completion of meiosis, resulting in extrusion of the first polar body. (b) Pronuclear envelope forms around the remaining oocytes chromosomes to give rise to the female pronucleus whilst the male chromosomes decondense forming the male pronucleus. Additionally a sperm aster resides in close proximity to the male pronucleus, from which a microtubule array extends towards the female pronucleus. (c) Female pronucleus is pulled towards the male pronucleus. (d) Pronuclear envelopes breakdown. (e) Here the centrioles migrate to opposite poles and the chromosomes align ready for the first mitotic division. (f) Chromosomes segregate into two new daughter cells. (Image adapted from original found in Feenan and Herbert, 2006).

1.5.6 Blastocyst development

The human cleavage stage embryo is a unique stage during pre-implantation embryonic development during which the cells exist in an undifferentiated state, still as yet to commit to a specified cell type. This state is often referred to as 'totipotency'. The blastocyst stage embryo however is a unique stage of pre-implantation embryonic development, during which cells begin to adopt a cell identity, which ultimately culminates in commitment to a defined cell lineage. There are two distinct cell types found in the mammalian blastocyst: the outer epithelial layer or trophectoderm (TE) and the inner cell mass (ICM) (Hardy et al, 1989) (Fig1.13a). The TE is responsible for blastocoel fluid accumulation and is also specialized for implantation (Hardy et al, 1989; Rossant, 2004). Upon implantation the TE gives rise to components of the placenta and extra-embryonic membranes The ICM however goes on to form the 'embryo proper' and gives rise to three germ layers of the foetus (Gardner and Papaioannou, 1975). The PE (primitive endoderm) and EPI (epiblast) cells make up the cells of the inner cell mass (ICM), those cells which lie within the blastocoel cavity.

1.5.6.1 Lineage specification

The allocation of cells to these two 'primary lineages is said to occur following the fourth cleavage division (Handyside, 1981; Hardy et al, 1989) Studies have shown that following commitment of cells to one of these two 'primary lineages' the transcriptional regulation of the cell is altered to allow for the precise expression of a subset of genes. The most important genes at this early stage of embryonic development are those involved in regulating the pluripotent state of cell. A number of genes have been identified which are solely expressed in extra-embryonic cells (TE) Cdx2 is a caudal-related homeodomain protein and a key regulator of the trophectoderm lineage (Rossant and Tam, 2009) (Fig1.13b). Studies have shown that Cdx2 begins to be expressed around the eight-cell stage of embryo development before its expression begins to become restricted and up-regulated in the outer cells of the morula, prior to blastocyst formation (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008; Rossant and Tam, 2009). Studies have demonstrated that the promoter of the Cdx2 gene is highly acetylated in the trophectoderm but enriched in H3K9me2 in the ICM (O'Neill et al, 2006). This therefore results in suppressed expression of Cdx2 within cells of the ICM

however as Cdx2 expression is not suppressed in the trophectoderm cell, we would expect to see if expressed widely in these cells.

Another key transcription factor critically involved in maintaining cells in an undifferentiated, pluripotent state is Nanog (Rossant and Tam, 2009; Ozawa and Hansen, 2011). Nanog is usually expressed ubiquitously in the cleavage stage preimplantation embryo. Its expression is gradually down-regulated as cell fate is adopted and consequently expression becomes restricted to a small number of cells found within the inner cell mass following blastocyst formation (usually around day 5-7) (Fig1.13b). A second transcription factor, Gata6, a zinc-finger transcription factor, was previously shown to be required for the development of extra-embryonic tissues, as demonstrated through the generation of chimeric embryos (Beddington and Robertson, 1989; Koutsourakis, et al, 1999). In the early to mid-stage mammalian blastocyst, the ICM still contains a heterogeneous population of cells, composed of pluripotent epiblast and extra-embryonic primitive endoderm (PrE) progenitors, which is often demonstrated by the "salt and pepper" distribution of the key epiblast (NANOG) and PrE (Gata6) markers (Koutsourakis et al, 1999; Rossant et al, 2003; Chambers et al, 2003, 2007; Mitsui et al, 2003; Chazaud et al, 2006; Silva et al, 2009) (Figure 1.19b). It is not until the late blastocyst stage, that compartmentalization results in the establishment of two distinct expression domains for Nanog and Gata6, delineating the newly formed epiblast and PrE lineages (Plusa et al, 2008; Meilhac et al, 2009; Lavial et al, 2012). Nanog and Gata6 mutually inhibit each other and thereby segregate the two lineages of the ICM (Lanner and Rossant, 2010). It has further been suggested that Gata6 has the ability to promote cellular properties that will allow correct sorting of PrE progenitors that are in the wrong position in the ICM (Rossant et al, 2003). This would create a model in which the EPI and PrE precursors are specified in a random manner within the ICM, inferring that it is not until a later time point that they segregate to their respective layers (Rossant et al, 2003; Chazaud et al, 2006; Lavial et al, 2012). Therefore if this "salt and pepper" like distribution is to be believed, it contradicts the previously assumed model of cell positioning which assumed that cell position with respect to the blastocyst cavity is the primary determinant of PrE fate (Yamaaka et al, 2006).

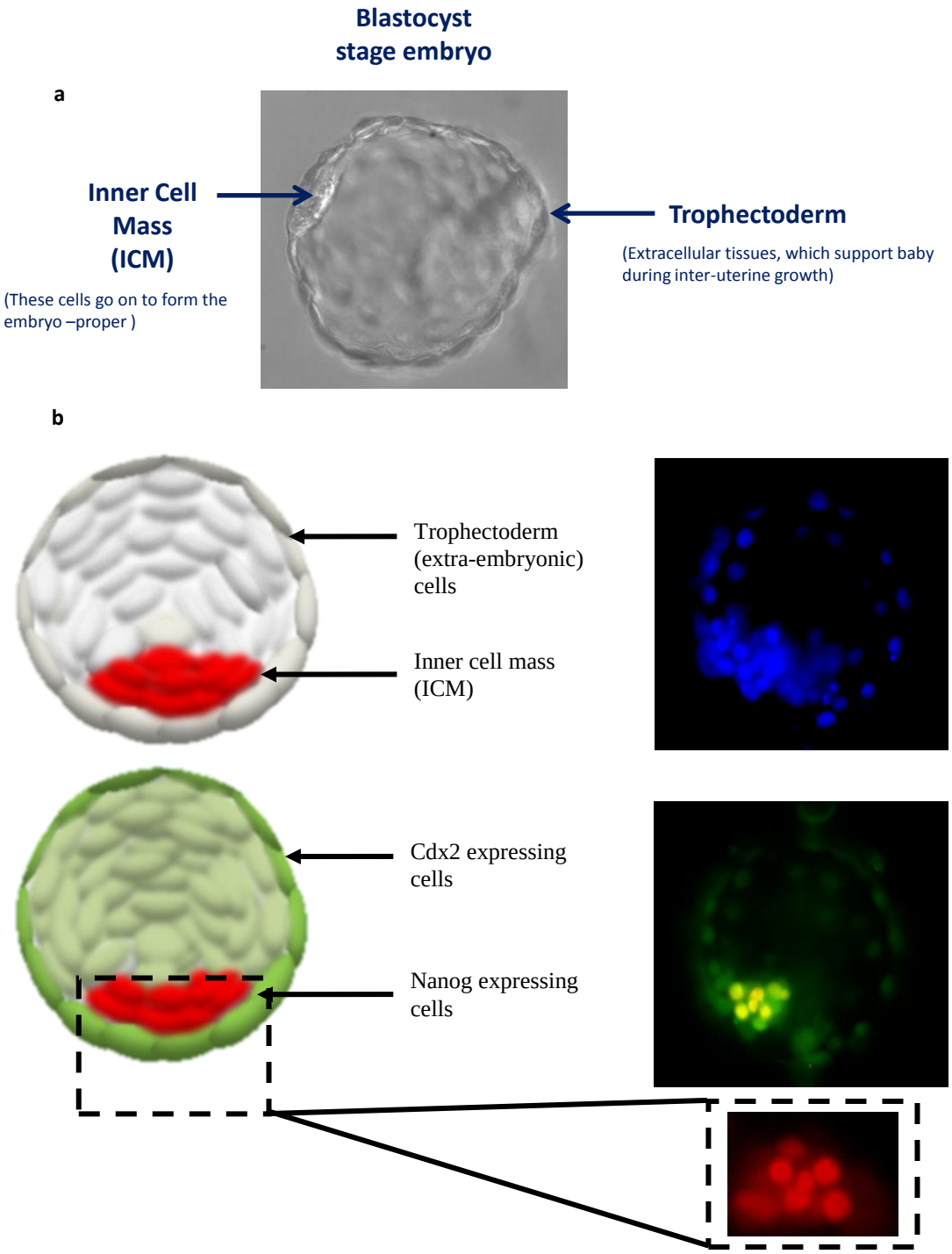


Figure1. 13 Cell lineage specification in the mammalian blastocyst

Above images represent different cell types within the pre-implantation blastocyst and stains used to identify these different cell lineages(a) a DIC image of a human day 7 hatched blastocyst, with labels marking the two cell types present at this point, the trophectoderm and the inner cell mass (ICM). (b) A schematic representation of blastocyst, depicting both the trophectoderm cells and the ICM and blastocyst stained using DAPI (nuclear stain-blue) and Nanog (cells which are still pluripotent in nature-red) (c) A schematic representation of a blastocyst, depicting the trophectoderm cells (green) and Nanog cells (red) and images from blastocysts stained for the markers Gata6 (green) and Nanog (red).

1.6 Cryopreservation strategies used to preserve fertility

Prior to the availability of cryopreservation technology, women producing excess oocytes had no choice but to discard excess non-transferred viable embryos (Veeck, 2003). However following the introduction of successful cryopreservation programmes, wastage of supernumerary embryos has and can be reduced as couples can instead choose to store excess embryos for future attempts (Trounson and Mohr, 1983; Valojerdi et al, 2009). Consequently embryo cryopreservation has become regarded as an integral part of the IVF process.

Cryopreservation also facilitates in the transfer of fewer embryos per cycle which can reduced the risk of multiple pregnancy per cycle. However the overall chance of conception and the cumulative pregnancy rate in an IVF programme can instead be enhanced by better utilising stored embryos for future frozen embryo transfer (FETs) cycles. Cryopreservation can also prove useful in situations when embryo transfer is unable to go ahead, for example in patients at risk of ovarian hyperstimulation syndrome or patients preparing to undergo radiation or chemotherapy (Shamonki and Oktay, 2005). However in order for successful outcomes it is imperative that a successful cryopreservation strategy is in place firstly.

Controlled-rate freezing has often proven to be the preferred option for embryo cryopreservation for the past decade within IVF laboratories. However In recent years an alternative cryopreservation technique, known as vitrification has been revisited. The greatest benefit which has resulted from the introduction of vitrification is the high recovery and survival rates which can be achieved for oocytes, embryos and blastocyst stage embryos once not possible using conventional controlled-rate freezing (Lucena et al, 2006; Ciotti et al, 2009; Capalbo et al, 2010; Rienzi et al, 2010). Both of these techniques will be described in greater detail in the following paragraphs.

1.6.1 Controlled rate freezing

Controlled rate freezing involves step-wise programmed decrease in temperature. The procedure is lengthy and requires the use of expensive instrumentation. However controlled-rate freezing has been successfully used to cryopreserve embryos at various different stages of embryonic development, including; pronuclear, cleavage and

blastocyst stage (Al-Hasani et al., 2007; Barg et al., 1990; Senn et al., 2000; Van den Abbeel et al., 1997a; Desai et al., 2007; Kuwayama et al., 2005; Kuwayama et al., 2005; Liebermann and Tucker, 2006; Menezo, 2004). However due controlled rate freezing is noutourisiously associated with the risk of ice crystal formation within the cells during the cryopreservation procedure. Consequently this can have extremely deleterious effects and often result in reduced survival outcomes (Pegg, 2005; AbdelHafez et al, 2010). Indeed the successful cryopreservation of oocytes using controlled-rate freezing has proven impossible. This is due to the high water content of oocytes which renders them prone to cryo-injuries as a result of the cryopreservation procedure (Toner et al. 1990, Ruffing et al. 1993, Arav et al. 1996, Zeron et al. 1999; Coticchio, 2007) .

1.6.2 Vitrification

However Vitrification literally meaning 'to turn to glass' frequently referred to as a novel technology of cryopreservation in embryology has been used as an alternative to 'controlled-rate freeing' for the past decade (Loutradi et al, 2008). Rall and Fahy were the first to report the successful vitrification of mammalian embryos back in 1985, with the first reported pregnancy from a vitrified embryo following in 1990 (Valojerdi et al, 2009). However there were several issues associated with the vitrification technique which resulted in controlled-rate freezing taking president as the preferred method of cryopreservation within IVF laboratories. This primarily concerned the solutions and devices employed to perform vitrification, which were sub-optimal and exposed embryos to high concentrations of cryoprotectants for prolonged periods of time in large volumes (Fig1.14).

Although in recent years there has been a resurgence of the vitrification technique with an increasing number of IVF clinics now opting to replace controlled-rate freezing cryopreservation strategies with vitrification based systems. This has been attributed to the vast improvements which have been made with regards to the solutions and devices used to perform the vitrification procedure. The majority of these solutions and devices have been designed with several key principles in mind which are fundamental to achieving successful cryopreservation outcomes, including; using a combination of cryoprotectants, employing multiple-step protocols, minimal loading techniques and

high cooling rates. Whereby ensuring these criteria are met, vitrification is able in many cases to guarantee survival.

The reinvention of vitrification has indeed revolutionized the cryopreservation of embryonic samples within IVF laboratories worldwide. Embryos are now able to be successfully vitrified at all stages of embryonic development resulting in improvements in clinical pregnancy rates for frozen embryo transfers compared to that once achievable with conventional controlled-rate freezing (Mukaida et al, 1998; El-Danasouri et al, 2001; Mukaida et al, 2003; Liebermann et al, 2002; Vanderzwalmen et al, 2002; Liebermann et al, 2004; Huang et al, 2005; Kumasako et al, 2005; Kuwayama et al, 2005; Raju et al, 2005; Zech et al, 2005; Liebermann and Tucker, 2006; Kuwayama, 2007; AbdelHafez et al, 2010). Huge leaps in survival outcomes have been observed for cleavage and blastocyst stage embryos which due to their high water content and large surface areas were often fraught by the risk posed from cryoinjuries, induced as a result of the sub-optimal conditions associated with controlled-rate freezing (Balaban et al, 2008; Papanikolaou, et al, 2008). Using vitrification it is also now possible to successfully cryopreserve oocytes once not feasible using controlled-rate freezing (this will covered in greater detail in chapter 6) (Lucena et al, 2006; Ciotti et al, 2009; Capalbo et al, 2010; Rienzi et al, 2010). This has proved extremely important for women requiring fertility preservation. As previously oocytes were often fertilised using either partner or doner sperm and subsequently cryopreserved at the single cell-zygote or cleavage embryo stage of embryonic development due to an inability to successfully cryopreserve oocytes. However this was often not ideal for young oncology pateints in particular who have not yet found a partner they wish to begin a family with; often having to resort to using donor sperm to create embryos which could be stored. However now they are able to cryopreserve oocytes which they can then use at a later date when they find a partner whom they want to start a family with.

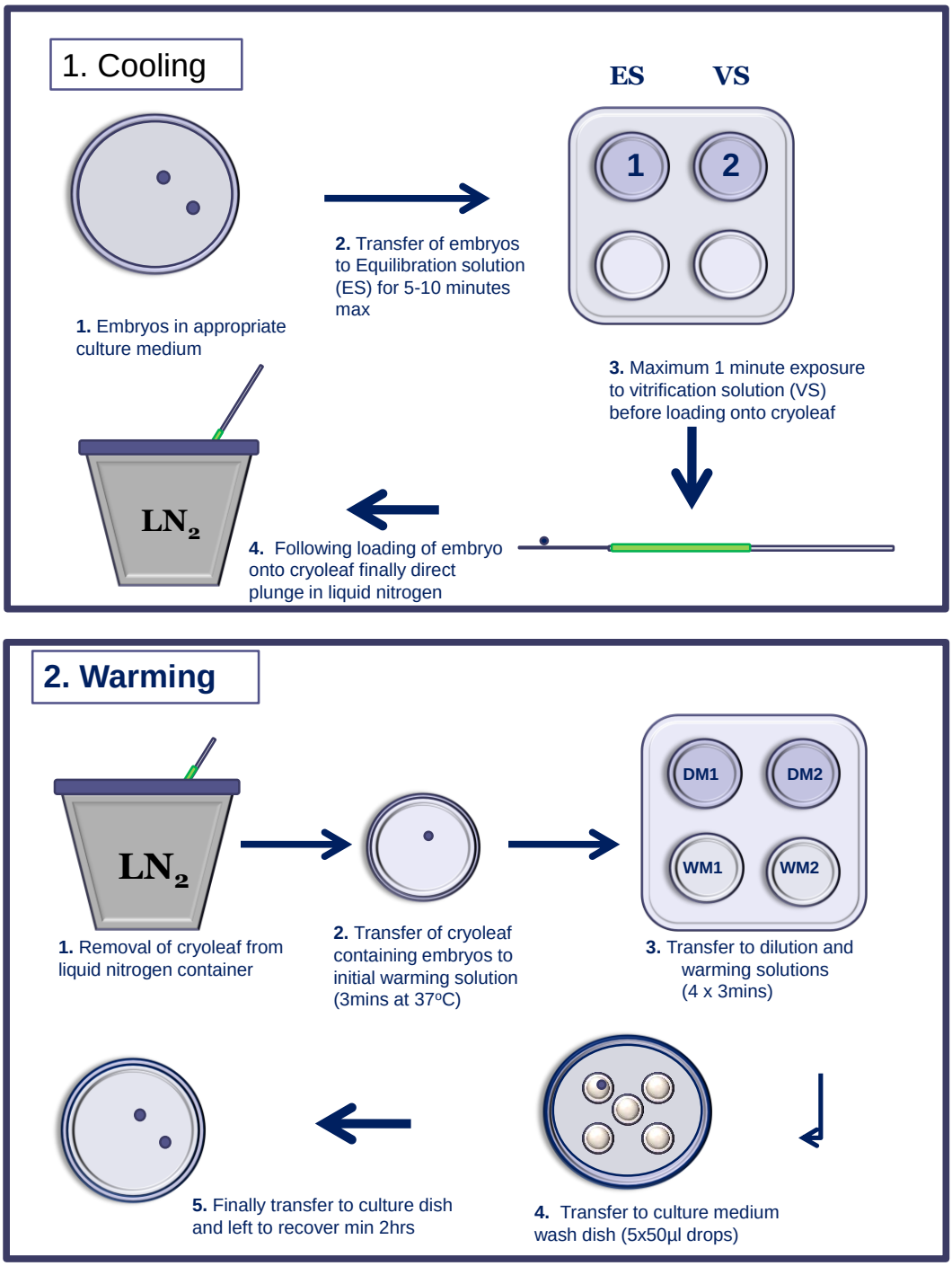


Figure1. 14 vitrification: cooling and warming

(a) Schematic representation of the cooling procedure. Here embryos are passed through a series of solutions, which act to prepare the oocyte before they are plunged rapidly into liquid nitrogen and stored for indefinite periods in liquid nitrogen storage tanks. (b) Schematic representation of the warming procedure - Here devices containing embryos are removed from liquid nitrogen storage before the device containing the embryos are transferred to the initial warming solution to begin the warming procedure. Embryos are then passed through a series of sequential solutions which act to remove the cryoprotectants gradually in a step-wise manner so as to not shock the embryos.

1.7 Current and future approaches for the treatment and prevention of mtDNA disease

Due to the lack of data regarding the rules of segregation of wild type and mutant mtDNA species and tissues in the developing embryo, it is difficult to predict the resulting phenotype of a child (Steffann et al, 2006). However there are several tests available which have the ability to assess the likelihood of a couple having a child affected by a life threatening disease. These tests should be explained to the couple through genetic counselling within the clinic. These include oocyte donation, prenatal screening, prenatal genetic diagnosis, and pre-implantation genetic diagnosis (DiMauro et al, 2001; Thorburn et al, 2001; Poulton et al, 2000; Poulton et al, 2002; Taylor et al 2005). Although these techniques have the potential to indicate the potential degree by which a child is likely to be affected by a disease, they are by no means error proof.

1.7.1 Genetic counselling

As previously discussed, mitochondrial DNA defects can be transmitted by maternal inheritance (Chinnery, 2006). Whilst the majority of mtDNA deletions occur *de novo* and therefore usually only affect one single family member harbouring no potential threat to other family members, point mutations can be transmitted down the maternal line. Therefore a mother harbouring a mtDNA point mutation may transmit a variable amount of mutant mtDNA to her offspring due to the phenomenon of heteroplasmy. This can result in considerable clinical variability amongst siblings within the same family. Therefore patients who have been found to harbour a pathogenic mtDNA mutation must be made fully aware of the future risk when considering a family and be made aware of the options available to them, including prenatal and preimplantation genetic screening as now discussed.

1.7.2 Oocyte donation

Oocyte donation is the process whereby a women consents for her eggs to be donated for use in reproductive purposes. Oocyte donation is therefore the equivalent to sperm donation, as both procedures depend upon the donation of gametes (Baetens *et al*, 2000). Oocyte donation is somewhat more controversial, as oocyte donation can

involve some invasive medical procedures, including ovarian stimulation and retrieval of mature oocytes under local anaesthetic (Baetens et al, 2000). Oocyte donation has become a standard treatment for women with diminished ovarian reserve, those who are of advanced reproductive age, women affected by or are carriers of a significant genetic defect, and women with poor oocyte quality (Cameron et al, 1989; Beatens et al, 2000; Cobo et al, 2010).

1.7.3 Prenatal genetic diagnosis

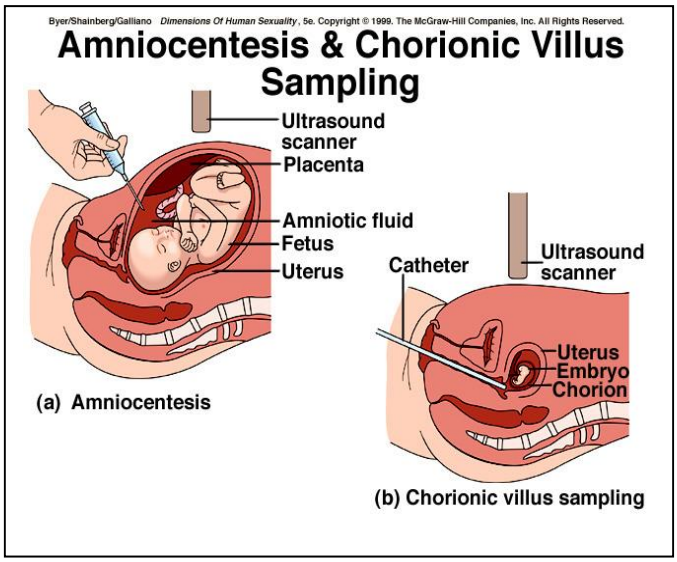
Prenatal diagnosis (PND) is generally defined as testing for diseases or conditions in a foetus or embryo before it is born. This can be performed as early as 11 weeks into the pregnancy by chorionic villus sampling (CVS) and as late as 20 weeks by amniocentesis however both of these are invasive procedures which can cause additional complications (White et al, 1999) (Fig1.15a).

PND is now available for mitochondrial disorders, however molecular analysis and interpretation of results is somewhat complicated by the phenomenon of mtDNA heteroplasmy (White et al, 1999; Chinnery, 2006). Heteroplasmy refers to the coexistence of at least two mtDNA molecules within one cell. This is also sometimes referred to as the 'mutant load' which is the proportion of mutant mtDNA molecules present within an individual (White et al, 1999). The ability to predict how a mutation may be transmitted to a future offspring is not only influenced by heteroplasmy but also due to the presence of a genetic bottleneck during pre-or postnatal development. There are also concerns that the mutant load may vary among tissues and may change over time, which has observed in adults with mitochondrial disease (Harding et al, 1992; Poulton and Morten, 1993; Poulton et al, 1998; Chinnery, 2006).

This has therefore raised concern that the percentage levels of mutant mtDNA observed in the extra-embryonic tissues sampled via PND may not truly reflect those levels present in the foetal tissues at birth (White et al, 1999; Bredenoord et al, 2010). However there are a limited number of studies which have reported a uniform distribution of mutation load among different tissues from two heteroplasmic 24-and 25-week-old human fetuses obtained from a carrier of the m.3243A>G mutation (Cardaioli et al., 2000; Matthews et al., 1994). Indeed PND has now been reported to have been performed successfully for a number of different mtDNA inherited mutations (Harding et al, 1992; White et al, 1999; Thorburn and Dahl, 2001; Chou et al, 2004;

Bouchet et al, 2006). Although one disadvantage to the PND procedure is that it is only able to be performed once a pregnancy has been established. Patients must therefore be aware that dependent upon results this may lead to a selective termination if the foetus is determined to have a high proportion of mutant mtDNA and they wish to prevent the birth of an affected child.

a



b

Figure1. 15 Procedures performed for prenatal genetic diagnosis (PND) and Pre-implantation genetic diagnosis (PGD)

(a) Images representing the processes performed to obtain a PND sample. This can be performed as early as 14 weeks in the pregnancy by chorionic villus sampling (CVS) and as late as 20 weeks by amniocentesis. However these are both invasive procedures, which so carry additional risks and complications. (b) Image represents the biopsy procedure, performed in order to obtain a single blastomere. Samples obtained from both procedures are then sent for diagnostic molecular analysis (Images taken from http://www.learningdigitally.org/Science/GeneticsSites.html).

1.7.4 Preimplantation genetic diagnosis

Preimplantation genetic diagnosis (PGD) is now conducted on a routine basis for couples who are at a heightened risk of transmitting a genetic or chromosomal abnormality to their children. This can include autosomal recessive, autosomal dominant and X-linked disorders (Thornhill et al, 2005).

PGD requires the use of assisted reproductive technology (ART) in order to create enough embryos which can go on to be analysed via genetic testing. This ensures that only those embryos which do not test positive for the specific condition tested for, are transferred back to the mother (Thornhill et al, 2005). Traditionally PGD is conducted on embryos which are cultured in vitro to the 6-8 cell stage (which us usually around day 3); at this stage it is possible to perform an embryo biopsy. This usually involves the removal of at least 1 blastomere, although it also possible to remove two blastomeres, however as the number of blastomeres removed is increased, the likelihood of impaired development also increases (Cohen et al, 2007; Goossens et al, 2008; A DeVos et al, 2009; Van de Velde, et al, 2000)(Figure 1.15b). Following blastomere biopsy, molecular analysis is then performed to determine which if any embryos can be returned. Generally the two least affected embryos are returned on day 5 or day 6 post-analysis (Thornhill et al, 2005) (Fig 1.16).

However trophectoderm biopsy is now increasingly being used in place of conventional cleavage stage biopsy, since the trophectoderm gives rise to the extra-embryonic tissues, this is regarded as a safer procedure (Dokras et al, 1990). This change of direction is also a result of the vast improvements which have been made in blastocyst culture and freezing, which together with the advantage of an increased number of cells available for analysis make this an ideal stage to perform PGD (Lalic et al, 2001; De Boer et al, 2002; McArthur et al, 2005; Schoolcraft et al, 2011).

PGD is a relatively new option for women who carry heteroplasmic mtDNA mutations and involves identifying suitable embryos that have undetectable or low levels of mtDNA mutation associated with a lower risk of the offspring developing severe disease. Indeed this technique has been successfully applied to a small number of maternally inherited mtDNA mutations (Steffan et al, 2006, Thorburn et al, 2009, Monnot et al, 2011, Treff et al, 2012, Sallevelt et al, 2013) but as experience is still

limited, more information is required to determine the suitability of PGD for different pathogenic mtDNA mutations (Fig1.16).

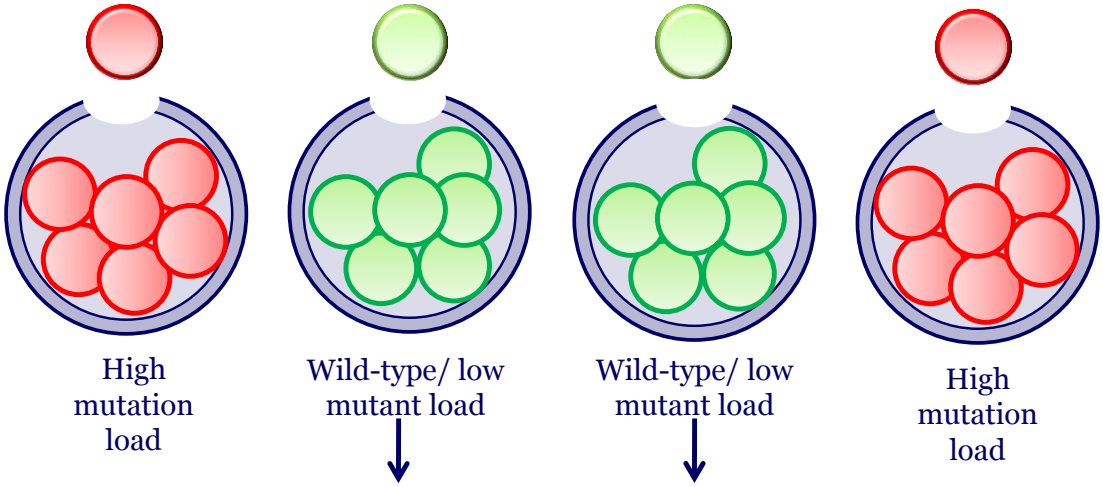


Figure1. 16 Preimplantation genetic diagnosis (PGD)

The diagram represents the process behind PGD. Here embryos are created by IVF, which are subsequently biopsied on day 3 and sent for molecular analysis. The results of this analysis determine which of the embryos are safe to be transferred back. A maximum of two embryos are able to be transferred back to the patient per cycle (Adapted from original image taken from Braude et al, 2002).

1.7.5 Currently explored IVF strategies to prevent the transmission of mitochondrial disease

Due to the lack of currently available treatments for mitochondrial disease and the unpredictable nature regarding mtDNA segregation during early embryonic development, several alternative strategies have been developed to try and help any at risk families. These are based upon nuclear transfer techniques, which involve the removal and transfer of genetic information providing a means by which couples could conceive healthy children free of mtDNA disease.

1.7.5.1 Germinal vesicle transfer

Germinal vesicle transfer is one particular nuclear transfer technique which has been proposed. This is similar to the pronuclear transfer technique whereby the GV is removed from the affected oocyte and transferred to the enucleated recipient oocyte (Spikings et al, 2006). Indeed studies using human oocytes have shown that GVT can overcome oocyte aneuploidy, with the majority of reconstructions having normal karyotypes (Zhang et al, 1999; Takeuchi et al, 2001; Spikings et al, 2006). However the success of GVT in human oocytes appears to be limited, as *in vitro* maturation following manipulation is fairly low (Zhang et al, 1999; Takeuchi et al, 2001). The *in vitro* maturation has been associated with essential removal of cumulus cells prior to manipulation procedures, as cumulus cells are considered important for oocyte maturation (Ge, et al, 2008). Therefore without improvements to in vitro culture conditions, it is unlikely that this technique could be used to prevent transmission of mtDNA disease.

1.7.5.2 Metaphase II spindle transfer

An alternative strategy proposed to prevent transmission of mitochondrial disease is ‘Metaphase II spindle–chromosomal complex transfer’ or ‘maternal spindle transfer’ as it is otherwise referred. Several studies have reported upon the MII spindle transfer procedure, which have so far been conducted in mouse, primate and human oocytes (Wang et al, 2001; Tachibana et al, 2009; Tachibana et al, 2010; Tachibana et al, 2012; Paull et al, 2012). Wang et al (2001) were the first to report the use of MII meiotic

spindle transfer performed in mouse oocytes. Metaphase II karyoplast transfer with human in-vitro matured oocytes has been proposed as a potential strategy to rescue aged oocytes (Tanaka et al, 2009). Here the authors demonstrated, using the ICSI technique, that these could be successfully fertilised and subsequently compatible with onward development as demonstrated by the blastocyst formation rates (Tanaka et al, 2009). Chromosome analysis was also performed upon a selection of the reconstructed oocytes following fertilisation and all analysed were found to contain normal diploid sets of 46 chromosomes, confirming the feasibility of the technique in human oocytes. A later study then reported upon the use of maternal spindle transfer for the preventing the transmission of mtDNA disease to offspring which they performed in mature non-human primate oocytes (*Macca Mulatta*) (Tachibana et al, 2009). Here they showed that the reconstructed oocytes were capable of supporting normal fertilisation, embryo development and producing healthy full term offspring. Subsequent genetic analysis confirmed that the nuclear DNA in the three infants originated from the spindle donor and the mtDNA from the egg donor. They were also able to demonstrate no detectable level of mtDNA carryover originating from the spindle donor (Tachibana et al, 2009) (Figure 1.17b). The same group later reported performing the same experiments this time using human oocytes (Tachibana et al, 2012). They were able to demonstrate the potential to perform MST in human oocytes however they did report observing abnormal fertilization for a number of embryos which underwent MST. Although the remaining embryos which were found to have undergone normal fertilization (as determined by the presence of two pronuclei) were capable of developing to blastocyst stage embryos. A second study published in the same year also examined the potential of MST in human oocytes. However they opted to use parthenogenetic activation instead of fertilization using ICSI, to overcome legality issues associated with creating embryos for the purpose of research. However they were still able to attain developmental efficiency to the blastocyst stage and undetectable levels of mtDNA carryover. Therefore providing further support for the MST technique, which they referred to as nuclear genome transfer to prevent the transmission of mtDNA disease in humans.

1.7.5.3 Pronuclear transfer

McGrath and Salter (1983) were the first to report the successful use of the pronuclear transfer manipulation technique which they performed in mouse embryos. They

described how using microsurgical apparatus they were able to remove the pronuclei from a donor mouse embryo and using a virus-mediated cell fusion technique reintroduce this into a previously enucleated recipient mouse embryo. Since this initial report, several groups have attempted to utilise the technique for different purposes. These include studies performed in mice to examine mitochondrial genotype segregation during preimplantation development (Meirelles and smith 1997; Meirelles and Smith 1998). One further study also examined the potential use of this technique as a possible method to prevent the transmission of mitochondrial disease to future generations in a mouse model (Sato et al, 2005). This technique has since been suggested as a possible method which could be used to prevent transmission of mitochondrial disease from mother to child in humans. This would involve the removal of both the female and male pronuclei from an embryo known to contain affected mitochondria to an enucleated, disease-free, donor embryo (Craven et al, 2010) (Fig1.17a). These studies performed in abnormally fertilised PN stage embryos were able to demonstrate that the transfer of pronuclei between human zygotes was indeed compatible with onward development and resulted in minimal carryover of mtDNA to the donor zygote (Craven et al, 2010).

1.7.5.4 Comparison between metaphase II spindle transfer and pronuclear transfer

Although the two techniques have each separately been shown to be technically feasible to perform and lead to low levels of carryover mtDNA, the question which has been asked is which is the better technique for patients with mtDNA disease (Craven et al, 2011). One other approach which may help in making this decision is to compare and contrast the two techniques, in order to fairly assess which approach should be favoured.

MST is performed using unfertilized oocytes, where only the female spindle chromosome complex is present. As a result the karyoplast produced using this technique is smaller, than possible with PNT, there is therefore potential for less carryover of mtDNA. However studies performed using both the MST and PNT technique have demonstrated equivalent levels of mtDNA carryover, indicating the risk

of offspring developing the disease is likely to be minimal (Craven et al, 2010; Tachibana et al, 2012; Paull et al, 2012).

However as the chromosomes of the metaphase II oocyte are not enclosed within a nuclear membrane concern has been raised that this could result in incomplete transfer of generic material to the recipient donor oocyte. At this stage the chromosomes are attached to a spindle which is only able to be visualized using polarized light birefringence (Oldenbourg, 1998; Liu et al, 2000; Konc et al, 2004; Rienzi et al, 2005; Montag et al, 2006). This is the only means by which the chromosomes can be visualized indirectly and safely without the use of a fluorescent dye, which could potentially affect future embryo viability and developmental potential. However there is evidence from studies performed in human oocytes that chromosome scattering can occur, which would not be able to be observed when using this imaging technique alone (Battaglia et al, 1996). Chromosome scattering has been associated with ageing and also sub-optimal environmental condition (Almeida et al, 1995; Cassimeris et al, 1988). It will therefore be important to ensure all experiments are performed with these points in mind. It may be that oocytes must be used for MST in the period immediately following oocyte retrieval and manipulations are performed in a controlled environment to overcome issues regarding spindle scattering.

PNT therefore offers the advantage of having the maternal and paternal genomes enclosed in clearly visible membrane bound structures, known as pronuclei. This overcomes issues associated with the MST technique regarding spindle integrity. However there are other problems which are associated with performing the nuclear genome transfer in the fertilized zygote. One such issue concerns the centrosome, which in humans is paternally derived (Sathananthan et al, 1991). It is possible that when removing the pronuclei from the patients zygote that the centrosome which is found tightly apposed to the male pronuclear membrane could be left behind, as a result of efforts made to reduce the size of the karyoplast the pronucleus is removed in. The centrsome plays an essential role in the first mitotic division, ensuring the assembly of a normal bipolar spindle (Sathananthan et al, 1996; Munne and Cohen, 1998; Craven et al, 2011). It will therefore prove important to ensure that the reconstituted zygote contains two centrioles. However by performing nuclear genome transfer before fertilization this issue can be avoided (Paull et al, 2012). Furthermore, pronuclear transfer requires the fertilization of both donor and recipient oocytes, resulting in the

destruction of half of the embryos. By contrast, human oocytes would only be fertilized after successful genome exchange (Paull et al, 2012).

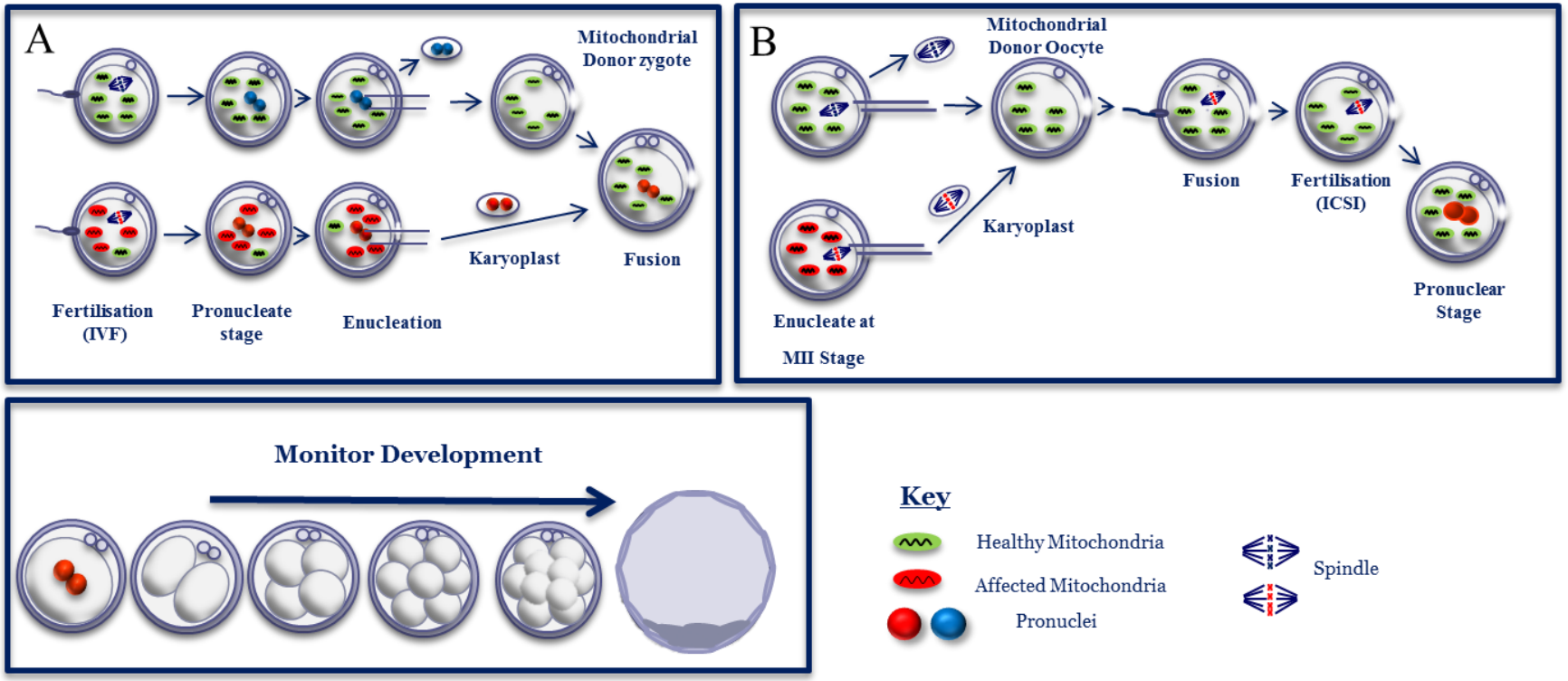


Figure1. 17 Comparison of pronuclear transfer versus metaphase II spindle transfer

The above diagram represents pronuclear transfer methodology. Here embryos are created by IVF, which results in the formation of two pronuclei one male and one female in origin if successful. The embryo from the mother harbouring the mtDNA pathogenic point mutation is then enucleated, whereby both the male and female pronucleus are removed using a biopsy pipette. Simultaneously a donor egg containing normal mtDNA is enucleated. The pronuclei from the affected egg are then transferred to the donor embryo and allowed to develop.

1.8 Ethical issues associated with the IVF based reproductive techniques

1.8.1 Ethical issues associated with Preimplantation genetic diagnosis (PGD)

Preimplantation genetic diagnosis (PGD) is an adjunct to asscited reproductive technology, and requires a patient to undergo ovarian stimulation in order to obtain oocytes or embryos for evaluation. The objective of the PGD technique is to screen for specific disorders in embryos from patients who are at risk of passing a life-limiting condition to their unborn child. Its main advantage is that it avoids selective pregnancy termination as the method used to select embryos makes it highly likely that the baby will be free of the disease under consideration. However since the first reported application of preimplantation genetic diagnosis (PGD) to the study of deleterious gene mutations in an embryo, there has been debate regarding the ethical acceptability of the IVF based procedure (Coutelle et al, 1989; Holding et al, 1989; Verlinsky, 2007).

There are two main sets of ethical objections against the use of PGD. The first arising from the requirement to create and then select embryos on chromosomal or genetic grounds, with the deselected embryos then usually discarded. The second concerns the process of selection itself (Robertson et al, 2003). The PGD technique also attracts religious objection and disapproval since some believe that it involves the destruction of human life.

The potential misuse of PGD has also been discussed extensively, with several different areas highlighted. Firstly the fear that PGD could be used for prenatal sexing of the embryo, and thus potentially used to select embryos of one sex in preference of the other in the context of "family balancing". A second fear is that it may prove possible to make other "social selection" choices in the future that introduce socio-economic concerns. A third concern is the potential to screen for genetic issues unrelated to medical neccesity, such as intelligence and beauty, and against negative traits such as disabilities. There is concern that this technique could result in the creation of ‘designer babies’ and this is often referred to as the ‘slippery slope’ argument.

Also there is concern regarding the reliability of the PGD technique, as PGD operates under the assumption that the mutation load observed in the cell removed at biopsy is representative of the mutation load in the remainder of the cells of the embryo. This may not be the case, as the incidence of mosaicism is often relatively high (Munne et al, 1998a). Therefore on occasion PGD may result in a false negative result leading to the acceptance of an abnormal embryo, or in a false postitive result leading to the de-selection of a normal embryo. However despite given these objections, there is a strong consensus that PGD is justifiable, at least for severe genetic disorders (Fasouliotis and Schenker, 1998; Buchanan et al, 2000; Verlinsky and Kuliev, 2000; Steinbock, 2002; Robertson, 2003, Klipstein, 2005; Kuliev and Verlinsky, 2005, a; Dresser, 2006; Bredenoord, 2008).

1.8.1.1 Ethical issues associated with the use of PGD for mtDNA inherited disorders

The use of PGD for mtDNA disorders raises several ethical questions in clinical practice (Bredenoord et al, 2008a, b, 2009). This is because diseases caused due to mtDNA-defects are more complicated to assess, due to the combined effects of heteroplasmy and the genetic bottleneck, which make it difficult to estimate the recurrence risks correctly and to provide an accurate prognosis for some mtDNA mutations (Bredenoord et al, 2008).

The concerns when performing PGD for mtDNA inherited disorders specifically relate to the effect of heteroplasmy. The possibility of having a large number of embryos with intermediate levels of mtDNA mutation loads results in what is deemed the 'grey zone' (Bredenoord et al, 2008). The exact threshold level of mtDNA mutation should be discussed during counselling of each individual couple, since it also depends on other factors such as disease manifestation and clinical severity on the family, perception of risk, the availability of embryos below the threshold and general fertility issues associated with IVF (Hellebrekers et al, 2012; Smeets, 2013)

A further strategy which has been suggested is the use of 'sex selection', for selecting male foetuses in order to eliminate the risk for future generations (Bredenoord et al, 2010). Sex selection in this particular approach is not intended to help a couple avoid having a child with a severe genetic disorder, but to avoid possible health risks in

further generations. Sex selection may be put to this use in the context of preventing mitochondrial DNA disorders by means of preimplantation genetic diagnosis (PGD) (Bredenoord et al, 2010). Sex selection against healthy female carrier embryos would have a two-fold advantage; firstly it would avoid difficult reproductive decisions for the future child and secondly avoid transmission of the mutation to a possible third generation. As Bredenoord et al (2011) writes, sex selection would still be done for reasons of health therefore this application should not give rise to the moral concerns associated with sex selection for non-medical reasons.

1.8.2 Ethical issues surrounding the nuclear transfer technique pronuclear transfer (PNT)

In view of the limitations posed by current reproductive techniques targeted at mtDNA patients (which at best are able to minimize the risk of mtDNA transmission from mother to child), the scientific community is searching for 'radical' alternatives, including ooplasmic transfer and pronuclear transfer (PNT). These approaches are often viewed as radical as they do not entail 'genetic selection' but instead 'genetic intervention' in order to correct the genetic cause of the disease (Bredenoord et al, 2009). There are a number of issues raised when considering the ethics of mitochondrial replacement techniques such as PNT.

1.8.3 Are PNT and other nuclear transfer techniques being explored for their ability to prevent the transmission of mtDNA disease, forms of cloning?

The question often posed when discussing the potential of the PNT technique and other nuclear transfer techniques aimed at preventing the transmission of mtDNA disease, is whether it amounts to 'human cloning'. This question can usually be answered both affirmatively and negatively depending on what is determined as the defining characteristic of cloning (De Wort, 2000; Jacobs et al, 2006; Bredenoord et al, 2008). Cloning is defined as the 'production of a genetic copy, genetically identical organism or individual' ('genetic duplication'). The procedure of cloning can be further classified as either; embryo cloning (when the original cell is derived from an embryo) or adult cloning (when the original cell is derived from an adult). However the aim of nuclear transfer procedures currently being explored is not targeted at the conception of

genetically identical individuals but at the prevention of severe genetic disorders. It therefore seems unreasonable to view such therapeutic nuclear transfer procedures as approaches to cloning.

1.9 Aims

- To examine mtDNA mutation loads in oocytes, embryos and blastomeres obtained from patients undergoing preimplantation genetic diagnosis (PGD).

- To learn and develop the skills to perform the pronuclear transfer technique.

  Examine potential modifications which could be made to the PNT technique to improve efficiency.

- To optimise a vitrification protocol for human zygotes and oocytes.

Chapter 2

Materials and Methods

Chapter 2 Materials and Methods

2.1 Collection of oocytes and embryos

Human oocytes and embryos were obtained from patients undergoing fertility treatment at the Newcastle Fertility Centre at Life following informed consent. The projects were licensed by the Human Fertilisation and Embryology Authority (HFEA) (see Appendix 1) and approved by the Newcastle and North Tyneside Local Ethics Committee.

2.1.1 Human Oocytes

Immature oocytes used in the study were obtained at the time of oocyte collection (day 0 of the IVF or ICSI cycle). These included both germinal vesicle (GV) stage oocytes, which were identified at the time of oocyte collection by a visible GV, and oocytes arrested at the metaphase I stage, which were identified by the absence of a first polar body. Mature oocytes used included freshly harvested metaphase II oocytes and metaphase II oocytes that failed to fertilise following intracytoplasmic sperm injection (ICSI) ~48hrs post-fertilisation. Failed fertilisation was determined by lack of visible pronuclei on day 1 of the IVF or ICSI cycle and failure to cleave by day 2. Immature oocytes were also cultured in G-FERT (Vitrolife, Sweden) at 37°C with 7% CO2 until they reached the metaphase II stage (determined by extrusion of the first polar body) and were used in the study.

2.1.2 Abnormally Fertilised Human Embryos

Embryos used in the study included abnormally fertilised embryos collected on day 1 of the IVF or ISCI cycle. These embryos were identified as abnormal by the presence of either 1 pronucleus (monopronucleate) or 3 pronuclei (tripronucleate) 18-19 hours after insemination. These embryos cannot be used in fertility treatment and are routinely rejected from the IVF program.

2.1.2.1 Blastomere biopsy

Embryo biopsy was performed using an inverted microscope (Nikon Eclipse TE2000-U) equipped with a micromanipulation system (Integra Ti, Research Instruments). To obtain individual blastomeres, embryos were placed in G-PGD medium (Vitrolife, Sweden) at 37^{o}C with 7% CO_2 and immobilised with a holding pipette. A small hole was made in the *zona pellucida* using a microsurgical laser (Saturn Active, Research Instruments) and a biopsy pipette used to gently aspirate the individual blastomeres. The blastomeres were transferred to sterile microfuge tubes for analysis and the biopsied embryo cultured in G2v5 Plus medium (Vitrolife) at 37ºC with 7% CO_2.

2.1.2.2 Whole embryo disaggregation to obtain individual blastomeres

Embryos were placed briefly in Acid tyrode's solution (<1 minute) to remove the *zona pellucida* and transferred to G-PGD medium. Blastomeres were obtained by continual pipetting of the embryo to disaggregate individual cells. Blastomeres were washed through several drops of PBS and transferred to sterile microfuge tubes for analysis.

2.2 Molecular Biology Techniques

2.2.1 DNA isolation

2.2.1.1 DNA extraction from single cells

Samples (Oocytes, embryos and blastomeres) were centrifuged at 13,000rpm for 10mins and 15µl cell lysis buffer (50mM Tris-HCl pH8.5, 1mM EDTA, 0.5% Tween-20, 200µg/ml proteinase K) added to each tube. The samples were incubated at 55^{o}C for 2 hours and at 95^{o}C for 10 minutes so as to inactive enzyme activity (method referenced Craven et al, 2010).

2.2.1.2 Follicular fluid DNA extraction

Follicular Fluid was collected at the time of oocyte retrieval and the ovarian cells pelleted at 13,000rpm for 10 minutes. The pellets were resuspended in a volume of ~200µl and stored at -20^{o}C until required. DNA extraction was completed using QIAsmp® DNA Mini Kit according to manufacturer's instructions (Qiagen). 20µl QIAGEN Protease was added to a 1.5ml centrifuge tube followed by 200µl Buffer AL. The sample was vortexed for 15seconds and incubated at 56^{o}C for 10 minutes. 200µl of 100% ethanol was then added and the sample vortexed for 15 seconds before being applied to a QIAamp Spin Column. The spin column was centrifuged at 13,000rpm for 1 minute and transferred to a clean collection tube. 500µl Buffer AW1 was added and the spin column centrifuged at 13,000rpm for 1 minute. Te spin column was transferred to a clean collection tube and 500µl Buffer AW2 added. The column was then centrifuged at 13,000rpm for 3 minutes and transferred to a clean collection tube. The DNA was eluted by adding 50µl Buffer AE and centrifuged at 13,000rpm for 1 minute (method referenced Craven *et al*, 2010).

2.2.2 PCR

In PCR reactions, 1µl of sample DNA was amplified in 25µl volumes containing 5µl, 5x reaction buffer, 1 unit Go Taq DNA polymerase (promega, UK), 3µl $MgCl_{2,}$ 2µM dNTPs, of 125ng each primer (100µM). PCR was performed with one cycle of 10min at 95^{o}C, 30 cycles of 45s at 95^{o}C, 45s at the appropriate annealing temperature (as detailed in figure), 45s at 72^{o}C, and finally followed by one cycle of 10mins at 72^{o}C. All standard PCR reactions were carried out on a Thermal Cycler (GeneAmp PCR system 9600). The annealing temperature differed depending on the primer pair and the extension time varied depending on the length of the amplified product. (A list of all primers is included in Appendix 2).

2.2.3 Gel electrophoresis, visualization and analysis

Four µl of loading dye was added to the PCR products, which were subsequently separated on 2% agarose gels (prepared by adding 1g agarose (Roche, diagnostics, UK) to 50ml1xTAE buffer (0.04M Tris-acetate, 0.01MEDTA), including 1.5µlethidium

bromide (l0mg/ml) (Sigma) to allow subsequent visualization]. A 1kb DNA ladder was loaded onto the gel to allow sizing of the DNA products. Electrophoresis was performed for 30-35 minutes in a electrophoresis tank (Bio-Rad) at 85V until adequate separation of the DNA was observed. The PCR products were then visualized and imaged using a UV light illuminator (Alphaimager™2200, Alpha Innotech).

Chapter 3

Analysis of mtDNA mutation loads in preimplantation embryos

Chapter 3 Analysis of embryos for pre-implantation genetic diagnosis for mitochondrial DNA point mutations

3.1 Introduction

Pathogenic mutations within mitochondrial DNA (mtDNA) are now commonly recognised as important causes of genetic disease (Elliot et al, 2008; Schaefer et al, 2008). MtDNA mutations are maternally inherited (Wallace, 2007) the risk of transmitting mtDNA disease is difficult to predict due to the co-existence of wild-type and mutant mtDNA within an individual (known as heteroplasmy). The presence of a genetic bottleneck during early development complicates matters further and results in considerable variation in the mtDNA mutation load transmitted to offspring. This makes genetic counselling extremely challenging and because of a lack of effective treatments, pre-implantation genetic diagnosis (PGD) is increasingly being offered to women who carry pathogenic mtDNA mutations (Figure 3.1). However unlike PGD performed for Mendelian inherited disorders, where the presence or absence of the specific mutation is detected from the nuclear DNA, PGD for mtDNA disease aims to detect the proportion of mutant mtDNA within the embryo which has limited its application. This technique therefore aims to identify those embryos with low mutant loads to be selected and has been used successfully for only a small number of mtDNA mutations (Steffan et al, 2006; Thorburn et al, 2009; Monnot et al, 2010). Therefore In view of the fairy limited evidence available for different mtDNA mutations I have looked at levels of heteroplasmy for a total of seven different mtDNA mutations in oocytes, blastomeres, inner cell mass (ICM), trophectoderm and whole embryos.

3.1.1 Mitochondrial segregation during pre-implantation development in mouse heteroplasmic embryos

Most available data regarding mtDNA segregation during pre-implantation embryo development has come from animal studies. Mouse strains have been constructed which are heteroplasmic for two neutral mtDNA polymorphisms (Jenuth et al, 1996; Meirelles and Smith, 1997). These studies have demonstrated that mtDNA polymorphisms are transmitted to the offspring with levels of heteroplasmy largely determined by random genetic drift. Resulting in identical levels of observed

heteroplasmy within individual blastomeres of cleaved embryos throughout each of the cleavage stages examined (Dean et al, 2003). However they did report wide variations in the proportion of mtDNA variants in cleaved embryos compared with levels on the mother (Dean et al, 2003). Similar dramatic shifts of mtDNA populations between mother and her off-spring have been reported in cattle models as well (Laipis et al, 1988).

3.1.2 Mitochondrial segregation studies in the human pre-implantation embryo

Few data exist on mtDNA segregation during early human embryonic development. Studies have instead used polymorphisms to mimic the study of pathogenic mtDNA mutations in human pre-implantation embryos (Steffann et al, 2005, 2006). Here reporting relatively little variation in mitochondrial heteroplasmy amongst most pre-implantation embryos (<8%) with variation observed in the distribution of polymorphisms among embryos from a given mother, judged to be fairly small (14-17%). Studies examining mutation load segregation for the NARP (m.8993T>G) mutation in pre-implantation embryos demonstrated the virtually identical distribution of mutant species between blastomeres, which they were able to examine for 3 different embryos (Steffann et al, 2005). Another study also examining the m.8993T>G mutation in individual blastomeres revealed limited variation between blastomeres ranging from 2-11% (Tajima et al., 2007). A study examining segregation of the m.3243A>G mutation also reported observing equal partitioning of the mutant species between blastomeres (Monnot et al, 2011). The observed inter blastomere stability of the mutant load, which has now been reported for at least two different mtDNA mutations has led to the suggestion that the embryonic heteroplasmy determined for a single-cell provides an accurate estimate of the mutant load of the whole embryo (Steffann et al, 2006; Craven et al, 2010; Monnot et al, 2011).

3.2 Background to mutations included in analyses

3.2.1 m. 8993T>C

NARP syndrome is most commonly associated with two point mutations m.8993T>G and m.8993T>C both located within the MT-ATP6 gene. The m.8993T>G is generally associated with a more severe clinical phenotype in comparison so the m.8993T>C variant which even at similar levels of mutation is generally associated with a milder and less severe phenotype. Individuals who present with clinical phenotypes reminiscent of NARP syndrome most frequently carry the mutation at high levels >80% and >75% for the m.8993T>C and m.8993T>G mutations respectively (Steffann et al, 2007). Due to these extremely high mutation levels the risk of recurrence for this particular mutation is often equally as high. Indeed several studies have reported extreme variances in mutation loads during embryofetal development although predominately for the m.8993T>G variant which is known to demonstrate characteristic skewed patterns of segregation (<10% or >80%)(Blok et al, 1997; White et al, 1999; Leshinsky-Silver et al, 2003; Steffan et al,2006; Steffan et al 2007). Indeed the first successful reported case of PGD for a mitochondrial disorder was reported for the m.8993T>G NARP mutation, which resulted in the successful birth of a healthy baby boy (Steffann et al, 2007).

3.2.2 m. 9176T>C

The m.9176T>C mutation is located within the MT-ATP6 gene, the same gene the two NARP associated mutations (m.8993T>G and m.8993T>C) are located. Indeed it has been shown to bare similar resemblance in clinical symptoms to the NARP syndrome mutations (Dionisi-Vici et al, 1998). However the m.9176T>C mutation is a less well characterized mutation and the mutation load threshold for clinical expression has therefore proven more difficult to judge. Although of those cases reported in the literature most patients presenting with severe symptoms generally possess mutation loads above 90% in various tissues (Jacobs *et al*, 2005).

3.2.3 m. 10158T>C

The m.10158T>C mutation is located in the MT-ND3 gene (NADH dehydrogenase subunit 3), which encodes a subunit of complex I. Mutations in the MT-ND3 gene are commonly associated with complex I deficient Leigh Syndrome (LS) and Leigh-like syndrome (Wong, 2007; Malfatti et al, 2007; Bugiani et al, 2004). Leigh syndrome is

usually fatal during infancy (Huntsman et al, 2005; Wang et al, 2009. This mutation does appear to demonstrate quite consistent patterns of segregation although clinically affected tissues in patients often display very high levels of the mutation. The consistent pattern of segregation observed for this particular mutation is encouraging for patients and its prospective suitability for PND and PGD.

3.2.4 m. *14487T>C*

The m.14487T>C is a mis-sense mutation found within the ND6 subunit of complex I of the mitochondrial respiratory chain. Similarly to the m.10158T>C mutation it is also associated with isolated cases of Leigh syndrome (LS) and or in addition to bilateral striatal necrosis (BNS) (Solano et al, 2003; Wang et al, 2009). Variable levels of heteroplasmy are often reported for carriers of the m.14487T>C mutation (65-95%) (Wang et al, 2009) where mutation loads >90% are associated with a disease phenotype and onset of clinical symptoms typical of Leigh syndrome (Santorelli et al, 1993; DiMauro and Schon, 2001; Wang et al, 2009). It has therefore been said to display a similar pattern of inheritance to the NARP mutations (m.8993T>G, m.8993T>C and m.9176T>C) which also only become clinically apparent at high levels of heteroplasmy (Tatuch et al, 1992; Oritz et al, 1993; Degoul et al, 1995; Mäkelä-bengs et al, 1995; Santorelli et al, 1997; White et al, 1999).

3.2.5 m. 14709T>C

The m.14709T>C mutations is a well characterised mutation which has been found in several families with patients presenting with a proximal myopathy and diabetes (Hao et al, 1995). In some family members ataxia can also be an important and disabling feature (Van Hove et al, 2008; McFarland and Turnbull, 2008; Lax, 2012). The mutation is interesting because there is usually a fairly high threshold for disease and the level of mutation is pretty evenly spread throughout the tissues (Meulemans et al, 2007). Previous studies have demonstrated a correlation between the amount of mutant mtDNA and clinical severity within family pedigrees (Hanna et al, 1995).

Figure3. 1 Procedure performed for Preimplantation genetic diagnosis (PGD)

Image represents the biopsy process, performed in order to obtain a single blastomere, upon which molecular analysis can be performed.

3.3 Materials and Methods

3.3.1 Collection of oocytes and embryos

3.3.1.1 Human oocytes

Immature oocytes were included as part of this study. These were obtained following oocyte collection (day 0 of the IVF or ICSI cycle) and included germinal vesicle stage (GV) oocytes identified at the time of oocyte collection by a visible GV, and oocytes arrested at the metaphase I (MI) stage, which were identified by the absence of a first polar body. Mature oocytes used included freshly harvested metaphase II oocytes and metaphase II oocytes that failed to fertilise following intracytoplasmic sperm injection (ICSI) ~48hrs post-fertilisation. Failed fertilisation was determined by lack of visible pronuclei on day 1 of the IVF or ICSI cycle and failure to cleave by day 2. Immature oocytes were also cultured in G-IVF (Vitrolife, Sweden) at 37°C with 7% CO2 until they reached the metaphase II stage (determined by extrusion of the first polar body) and were used in the study.

3.3.1.2 Human Blastocysts

Following blastomere removal (see section 2.1.3.1), biopsied embryos were cultured *in vitro* to the blastocyst stage (day 5-7) and used in the study. Embryos not suitable for transfer or cryopreservation were cultured in G1 medium (Vitrolife, Sweden) at 37°C with 7% CO2 until the 8-cell stage (day 3 of the IVF or ICSI cycle) and were then transferred to G2 medium (Vitrolife, Sweden) at 37°C with 7% CO2 until they reached the blastocyst stage (day 5-7).

3.3.1.3 Cleavage stage thawing

Straws were removed from the liquid N_2 and held in the air for 30 seconds. Straws were incubated at 37^{o}C for 1 minute. The end of the straw was snipped off using sterilised scissors, and the contents were transferred to a 4-well nunc dish (NUNC, Thermofisher, Roskilde, Denmark). Using a denudation pipette embryos were

transferred to 500µl of TS1 (thawing solution) and left for 5 minutes at room temperature. Embryos were then transferred to the next solution TS2 (500µl) and left for a further 5 minutes, TS3 for 10 minutes and finally TS4 and incubated at 37^{o}C in a non-CO_2 incubator for 10 minutes. The thawed embryos were moved using a denudation pipette to a pre-equilibrated culture dish (37^{o}C and 7% CO_2).

3.3.2 Individual Blastomeres from Human Embryos

3.3.2.1 Blastomere biopsy

Embryo biopsy was performed using an inverted microscope (Nikon Eclipse TE2000-U) equipped with a micromanipulation system (Integra Ti, Research Instruments). To obtain individual blastomeres, embryos were placed in G-PGD medium (Vitrolife, Sweden) at 37^{o}C with 7% CO_2 and immobilised with a holding pipette. A small hole was made in the *zona pellucida* using a microsurgical laser (Saturn Active, Research Instruments) and a biopsy pipette used to gently aspirate the individual blastomeres. The blastomeres were transferred to sterile microfuge tubes for analysis and the biopsied embryo cultured in G2v5 Plus medium (Vitrolife) at 37ºC with 7% CO_2. Embryo biopsies were performed by Mrs Sam Byerley, Dr Lyndsey Butterworth and myself.

3.3.2.2 Whole embryo disaggregation to obtain individual blastomeres

Embryos were placed briefly in Acid tyrode's solution (<1 minute) to remove the *zona pellucida* and transferred to G-PGD medium. Blastomeres were obtained by continual pipetting of the embryo to disaggregate individual cells. Blastomeres were washed through several drops of PBS and transferred to sterile microfuge tubes for analysis. This was performed by Dr Lyndsey Butterworth and myself.

3.3.3 Samples included in analysis

After receiving informed consent from the donors, we obtained a number of samples from carriers of the m.3688G>A, m.3243G>A, m.8993T>C, m.9176T>C,

m.10158t>C, m.14487T.C, m.14709T>C mutations, all undergoing fertility treatment at the Newcastle Fertility Centre at Life. The project was licensed by the Human Fertilization and Embryology Authority (HFEA) and approved by the Newcastle and North Tyneside Local Ethics Committee.

3.3.3.1 m. 3688G>A

The samples included a previously biopsied cleavage stage embryo which was able to be disaggregated into individual blastomeres (n=12). Additionally several whole blastocysts (n=4) and a single blastocyst which was able to be successfully to give individual inner cell mass (ICM) and trophectoderm samples.

3.3.3.2 m. 8993T>C

Samples included two embryos, which blastomere biopsy revealed harbored high levels of mutation (>90%) and later went onto subsequently arrest development. Unfortunately both embryos contained considerable fragmentation and individual blastomeres were unable to be obtained and therefore embryos were collected as a whole for mutation load analysis.

3.3.3.3 m. 9176T>C

Here samples included two embryos biopsied on day 3, which arrested development on day 5. Both embryos were disaggregated manually and individual blastomeres obtained for both (n=5 and n=6).

3.3.3.4 m. 10158T>C

Samples included two embryos which had undergone blastomere biopsy and arrested development to be obtained (these were analyzed as whole embryos). An in vitro matured MII oocyte which had undergone polar body biopsy was also acquired for analysis.

3.3.3.5 m. 14487T>C

A number of frozen embryos were obtained from a carrier of the m.14478T>C mutation who had previously undergone fertility treatment at the Regional Fertility Program, Canada (Hewko, Calgary, Canada). A total of 5 embryos frozen at the 8-cell stage of development were thawed and cultured. One embryo was cultured to the blastocyst stage (day 7) and the trophectoderm and *zona pellucida* collected for mutation load analysis. The remaining embryos failed to reach the blastocyst stage and arrested development between the 8 to 18-cell stage (Fig3.2). These embryos were disaggregated into individual blastomeres for analysis.

3.3.3.6 m. 14709T>C

Samples included immature oocytes (GV and metaphase I stage) and a mature oocyte that failed to fertilize following IVF (metaphase II stage). A total of 3 fertilized embryos not suitable for transfer or cryopreservation were cultured to the 6-8 cell stage (day 3) and an embryo biopsy performed to remove a single blastomere for mutation load analysis. Two embryos were subsequently cultured to the blastocyst stage (day 7) and the trophectoderm and *zona pellucida* collected for analysis. The remaining embryo arrested development prior to the blastocyst stage and was disaggregated into individual blastomeres for analysis.

3.3.4 Single cell lysis

Individual oocytes and embryos were lysed for 2 hours in a standard lysis buffer (50mM Tris-HCl, pH 8.5, 1mM EDTA, 0.5% Tween-20 and 200µg/ml proteinase K) at 55ºC. The enzyme was inactivated by incubation at 95ºC for 10 minutes.

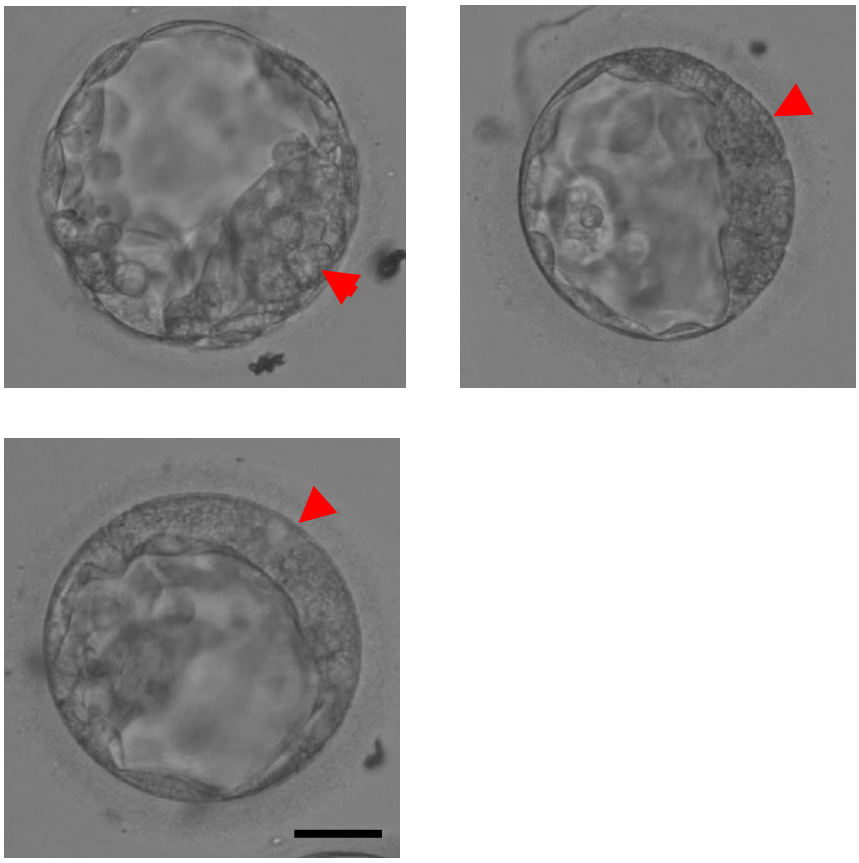


Figure3. 2 Thawed embryos analysed for the m.14487T>C mutation which successfully developed to blastocyst stage embryos

Above images represent embryos thawed at the 8-cell stage. These were then cultured and a number (n=4) successfully developed to blastocyst stage embryos (between days 5-7). These were then manually dissected so as to separate the inner cell mass (ICM) from the remaining trophectoderm cells. All blastocysts were found to contain relatively large ICM (as indicated by arrows), which assisted when performing the manual dissections of the blastocysts. Scale bars, 50µm.

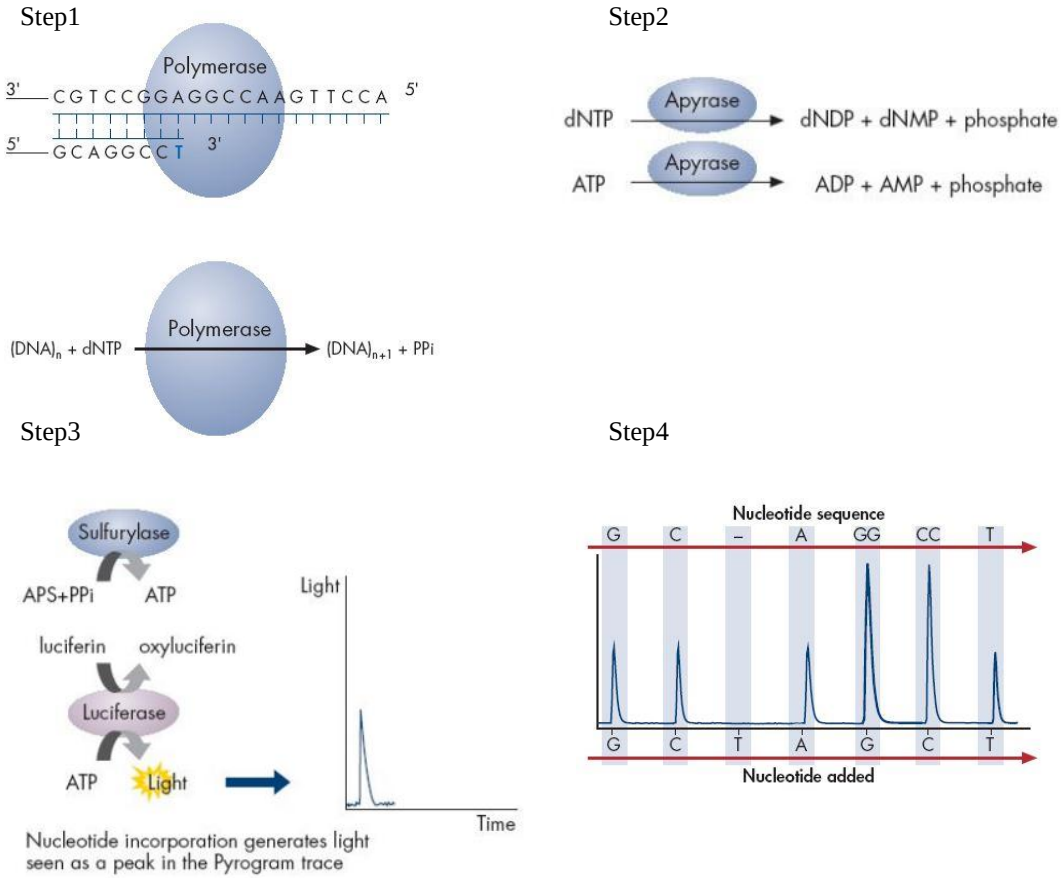


Figure3. 3 Above schematic diagram represents the principle methodology behind pyrosequencing

Here (step 1) the sequencing primer anneals to the single stranded PCR amplicon. Every time a new nucleotide is incorporated into the sequence a molecule of pyrophosphate is released (step 2). The pyrophosphate molecule released reacts with APS through a reaction catalysed by sulfurylase resulting in the production of ATP. The ATP generated then reacts to with luciferase supplied in the reaction reagents in so resulting in the generation of light (Step 3), which is detected and represented as a peak on the program trace generated (Step 4) (The height of each peak is proportional to the number of nucleotides incorporated at that specific part of the sequence being processed at the time. (Adapted from original images at www.qiagen.co.uk)

3.3.5 Pyrosequencing

We developed specific pyrosequencing assays employing a mutation-specific pyrosequencing primer to detect and quantify samples for the m.3688G>A, m.3243A>G, m.8993T>C, m.9176T>C, m.10158T>C, m.14709T>C and m.14487T>C mtDNA mutations. Primers were designed using PyroMark Assay Design Software version 2.0 Assays were designed by myself and Mrs Charlotte Alston.

PCR amplicons were generated from single cell lysate. Reactions were 25µl in volume and contained 1µl cell lysate, 5µl 5x reaction buffer, 1 unit Go Taq DNA polymerase (Promega, UK), 3µl $MgCl_2$, 2µM dNTPs and 125ng forward and reverse primer (100µM)(Please refer to, Appendix 2 Supplementary table1for full list of primer sequences). PCR reactions were performed on a Thermal Cycler (GeneAmp PCR system 9600) using the following amplification conditions: 95^{o}C for 10 minutes, followed by 30 cycles of 95°C for 45 seconds, annealing temperatures were assay specific for 45 seconds and 72°C for 45 seconds. The final extension was at 72°C for 10 minutes.

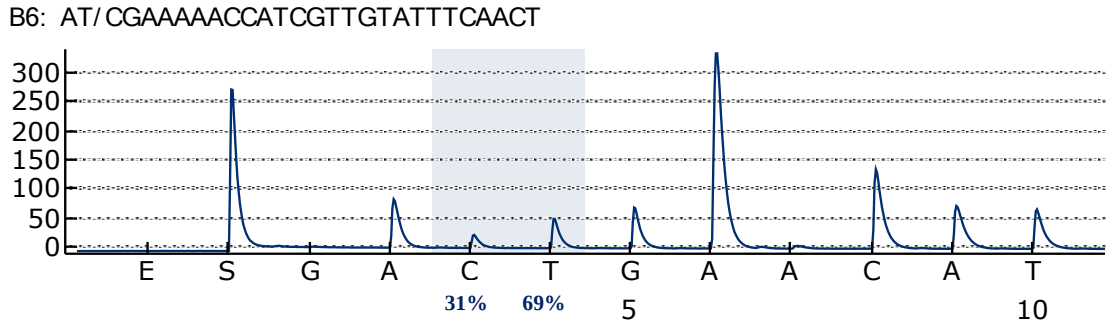
Pyrosequencing assays were performed in triplicate using the PyroMark Q24 instrument according to the manufacturer's instructions (Qiagen) (Figure3.3). Mutation load was quantified using the allele quantification PyroMark™Q24 Software (Figure3.4). To determine the sensitivity, specificity and accuracy of the assays, patient samples with known levels of the specific mutation previously quantified by hot last cycle-PCR RFLP were included in the analysis.

3.3.5.1 Validation of pyrosequening assays for the determination of mutation loads in preimplantation embryos

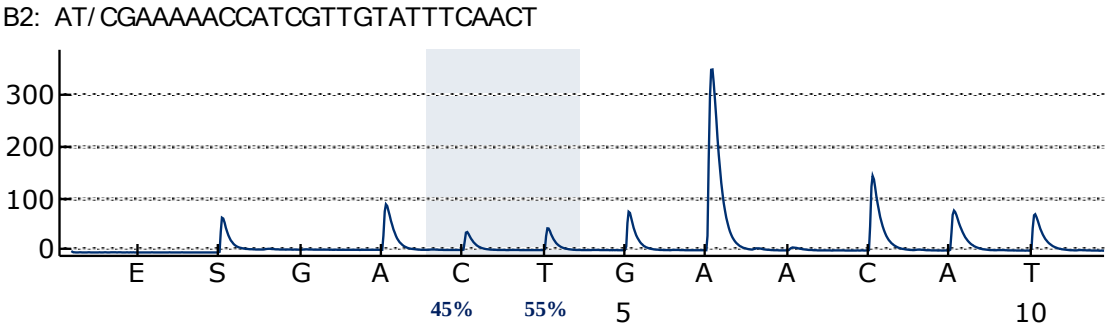
Pyrosequencing on the Pyromark Q24 platform was used to quantify the mtDNA mutations. PyroMark assay design software v2.0 (Qiagen, Hilden, Germany) was used to design mutation-specific pyrosequencing (PSQ) primer trios (as detailed in supplementary table). In order to determine the accuracy of the pyrosequencing assays, samples for which mutation load had previously been quantified using last hot-cycle RFLP were examined for each specific pyrosequencing assay. These samples were of

varying levels of heteroplasmy and with homoplasmic mutant mtDNA. Control samples were also included within runs from patients who were known to not carry the mutation of interest (WT-wild-type). These samples were also included in runs when analyzing embryonic samples to ensure assay accuracy. The allele quantification application of Pyromark's proprietary Q24 software was used to calculate mtDNA heteroplasmy levels.

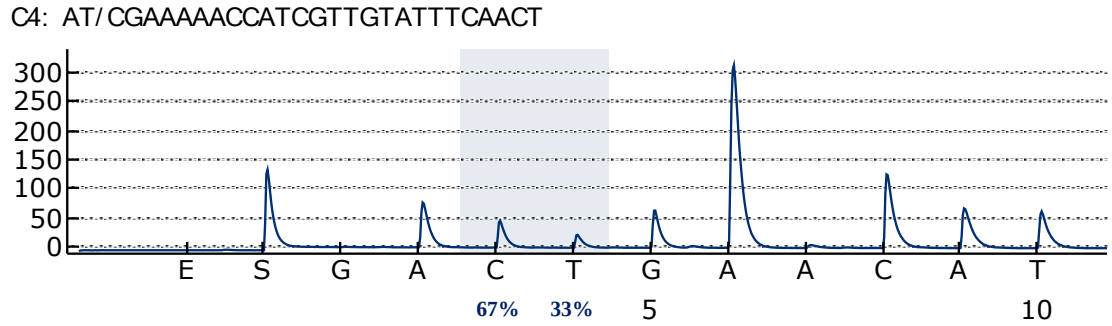
Metaphase II stage oocyte



Carrier of 14709T>C (Mum)



Heteroplasmic Mutant (Internal control)



Wild-type

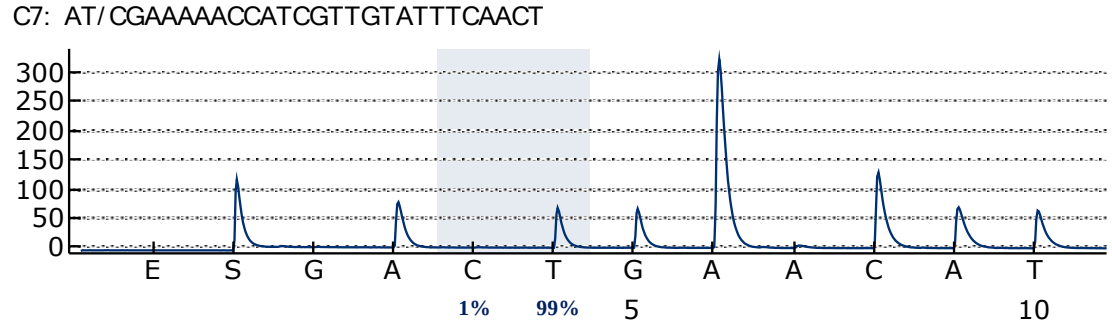


Figure3. 4 Pyrograms generated following pyrosequencing to determine mutation loads.

In each assay a number of internal controls specific to each assay were included. This included where possible a sample obtained from the mother which had previously been quantified, heteroplasmic mutant and wild-type sample were included to ensure correct validation and accuracy through consecutive runs.

3.4 Results

3.4.1 Mutation load analysis conducted for embryonic samples obtained from a carrier of the m.3688G>A mutation

3.4.1.1 Studies examining mtDNA mutation loads in embryos

Several samples were obtained from a patient recently confirmed as a carrier of the m.3688G>A. Previously the first child born to this patient died aged 3 months, from a complex I deficiency and Leigh syndrome, leading to an establishing a diagnosis of the m.3688G>A mutation in the mother. She was determined to carry the mutation load at 20% and 50% in blood and urine samples respectively, whilst her son was found to harbour the mutation at ~98%. Knowing the risk of potentially conceiving a second child also harbouring similarly high levels the couple opted to undergo pre-implantation genetic diagnosis (PGD). Several samples which were left over from her treatment programme and were not suitable to be cryopreserved were obtained via informed consent for mutation load analysis. This included a cleavage stage embryo biopsied on day three as part of the PGD programme here the mutation load was determined for the biopsied blastomere (4%) by the NCG mitochondrial diagnostic service. The embryo later arrested development and individual blastomeres were manually disaggregated (n=12 blastomeres) with mutation loads ranging from 1-7%, (mean= 5%) (Fig3.5). A number of the biopsied embryos successfully developed to blastocyst stage (n=5).

3.4.1.2 Studies examining mutation loads in blastocyst stage embryos

Here mutation levels were determined for the biopsied blastomeres obtained from three embryos on day 3 (by the NCG mitochondrial diagnostic service) as 16%, 8% and 2% and later for the corresponding blastocysts which were not transferred back to the patient (n=3) as 4, 6 and 3% (Fig3.15) (Table3.6). It was possible to manually dissect the inner cell mass (ICM) from the surrounding trophectoderm cells for one of the embryos which successfully developed to a blastocyst. Here the mutation load was determined for the ICM as 2% and the trophectoderm cells also as 2% (Fig3.6b). To

our knowledge this the first study we know of, which has attempted to successfully examine mutation loads in separate ICM (goes onto form embryo-proper) and trophectoderm (extra-embryonic tissues) samples. This was performed in order to determine if any variation in mutation loads existed between the two cell types. However no apparent preferential segregation was observed, although mutation loads were relatively low compared to wild-type levels. The mutation load was also determined for one blastocyst as a whole (3%) (Fig3.6a). No further information was available for this embryo as the biopsied cell acquired on day 3 failed to amplify.

3.4.1.3 Studies examining mtDNA mutation loads in oocytes

Mutation loads were also analysed for several oocytes obtained which were either found to be immature at egg collection or failed to fertilise (n=3). Here mutation loads were determined as 13% in the GV stage oocyte (Fig3.7a), 4% in the metaphase II stage oocyte (* with large polar body) (Fig3.7b) and 3% in the metaphase I stage (*zona*-free oocyte) (Fig3.7c). The corresponding empty *zona pellucida* was also included in analyses and mutation load determined as 42%* (*however this was attributed to cumulus cell attachment).

3.4.1.4 Studies examining mtDNA mutation loads for polar body biopsied oocytes

Two MII stage oocytes from the carrier of the m.3688G>A mutation which failed to fertilise and underwent polar body biopsy (performed by Ms Byerley) were also analysed. Mutation loads of 4% and 2% were observed in each polar body and the respective oocytes were determined as 3% and 3% (Fig3.8a and b)(Table3.1). Representing variation of ~1% between polar bodies and there corresponding oocyte, without taking into consideration errors in PCR and pyrosequencing.

m. 3688G>A

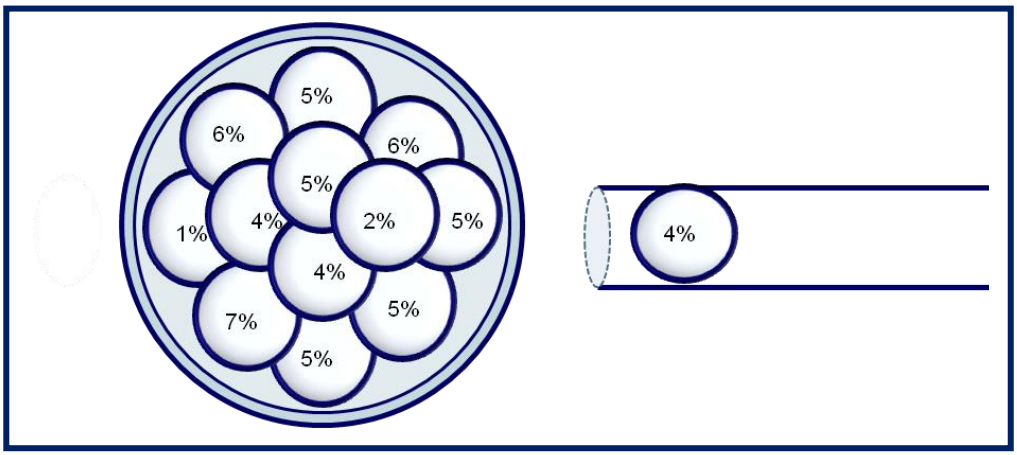


Figure3. 5 Analysis of mutation loads for individual blastomeres obtained for the m.3688G>A mutation

Above schematic diagram represents a cleavage stage embryo obtained from a carrier of the m.3688G>A mutation (12-cell stage) which subsequently arrested development and was able to be disaggregated into individual blastomeres (n=12). The mutation load was subsequently determined for the individual blastomeres with mutation loads ranging from 1-7%, (mean= 5%). This embryo had previously been biopsied on day 3 as part of the PGD programme (run between the Newcastle upon Tyne NHS Fertility Centre at Life and the NCG mitochondrial diagnostic service). Here the mutation load had been determined as 4% (analysis performed by Mrs C. Alston).

m. 3688G>A

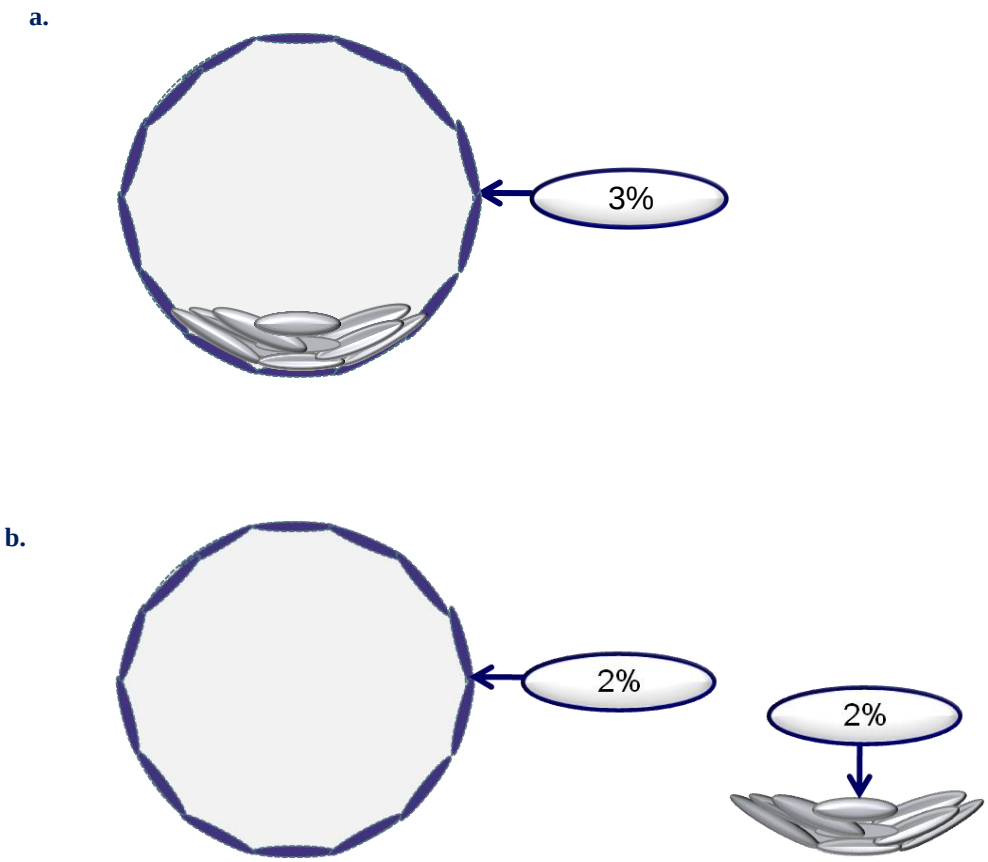


Figure3. 6 Determination of mutation loads in pre-implantation blastocysts for the m.3688G>A mutation

Two embryos which were biopsied on day 3 as part of the PGD programme for the m.3688G>A mutation but failed to amplify, so were excluded from the treatment cycle, successfully developed to blastocyst stage embryos. Here mutation load was determined for the whole blastocyst. (a) ~3% (b) for the second blastocyst the inner cell mass (ICM) and the trophectoderm cells were able to be dissected apart manually and collected for mtDNA mutation load analysis. Here the mutation load was determined for the ICM (2%) and trophectoderm cells (2%).

m. 3688G>A

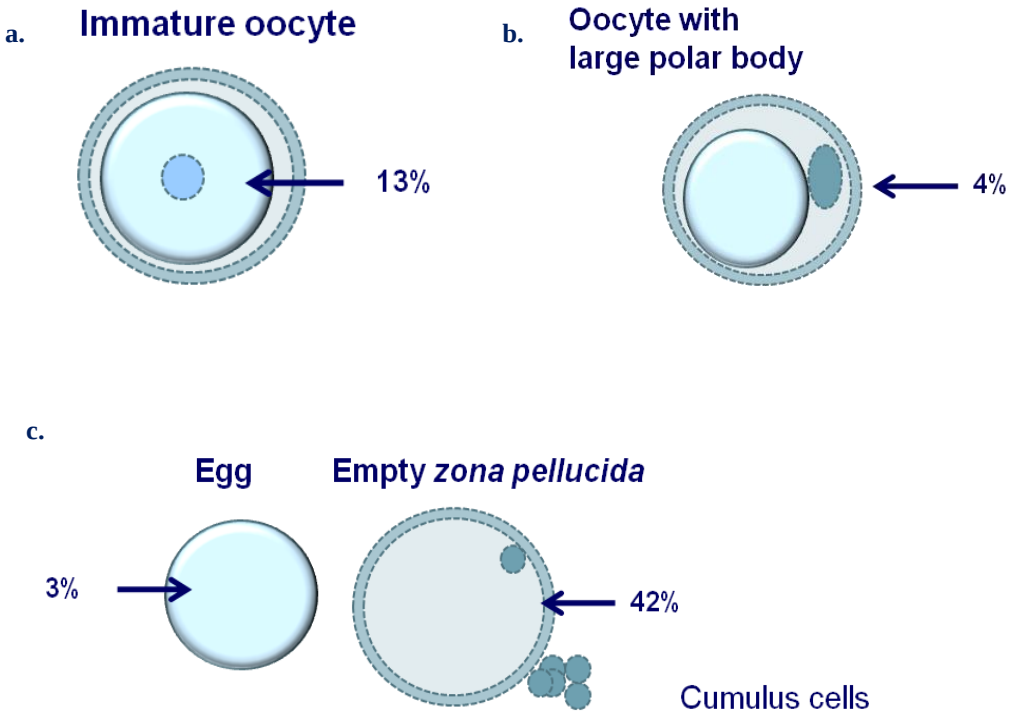
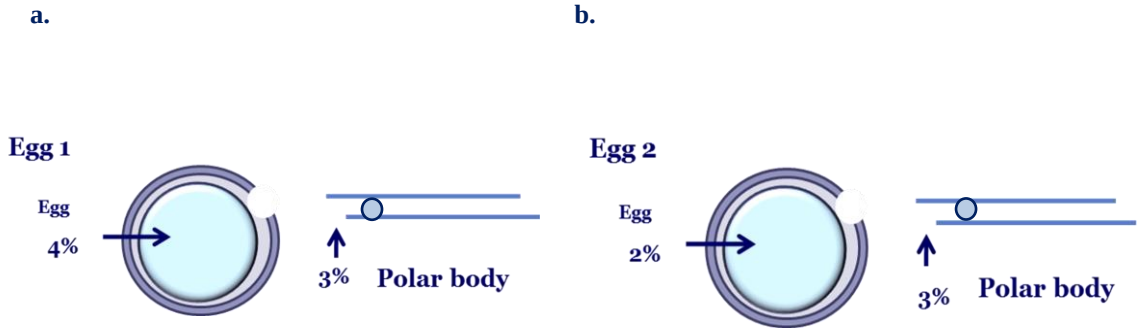


Figure3. 7 Schematic diagrams represent mutation load determined in samples obtained from a carrier of the m.3688G>A mtDNA mutation.

(a) Mutant load was determined for an immature oocyte (13%). (b) Metaphase II stage oocyte with large polar body (~4%) (c) Zona free oocyte (3%) and also corresponding empty zona pellucida (~42%). Here we believe the mutation load determined for the empty zona pellucida was influenced by the presence of cumulus cells which we know (following analysis) contained a higher mtDNA mutant load.

m. 3688G>A



m.10158T>C

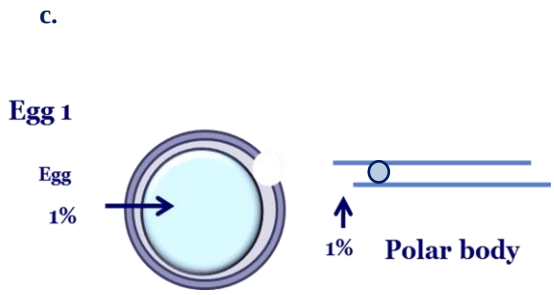


Figure3. 8 Schematic diagrams represent mutation load determined for two MII stage oocytes which underwent polar body biopsy from a carrier of the m.3688A>G mtDNA mutation.

For the two oocytes obtained from the carrier if the m.3688A>G mutation the oocyte mutant load was determined as (a) 4% and (b) 2% whilst the mutant load in the respective polar bodies was determined as 3% for both. For the m.10158T>C mutation (c) a single MII oocyte had a mutant load of 1% and this corresponded to the polar body which had an identical mutant load of 1%.

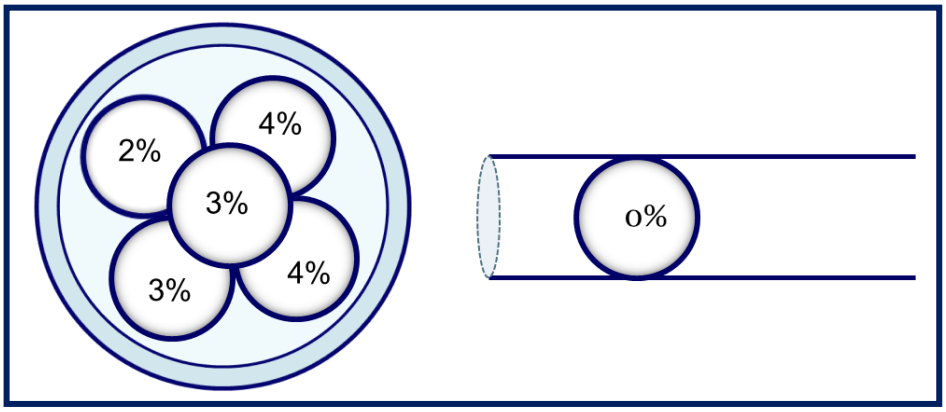
3.4.2 Mutation load analysis conducted for embryonic samples obtained from a carrier of the m.9176T>C mutation

3.4.2.1 Studies examining mtDNA mutation loads in embryos

Several samples were obtained from an asymptomatic carrier of the m.9176T>C mutation who previously had one affected child known to carry the mutation at high levels (>97%) and therefore opted for PGD to minimise the likelihood of conceiving another child affected by such high levels. Indeed previous studies have reported cases where maternal relatives of the patients either do not carry the mutation or carry the mutation at varying percentages (Jacobs *et al*, 2005). Here a number of embryos were created following IVF treatment. Those embryos which successfully fertilised were biopsied ideally on day 3 (p.f) involving the removal of a single cell. A number of these embryos arrested development (n=2) following biopsy procedures. Here the mutation was found to be undetectable in the biopsied cells (0%) (Mutation loads of blastomeres analysed by Mrs C. Alston as part of the NCG mitochondrial diagnostic service). Individual blastomeres were disaggregated for the arrested embryos (n=2) and mutation loads determined for each (1-4% and 2-4% respectively) (Fig3.9). Relatively low levels were detected in both of the embryos over all, which correlated with previous reports for mutation loads observed in the biopsied blastomeres.

m. 9176T>C

A



B

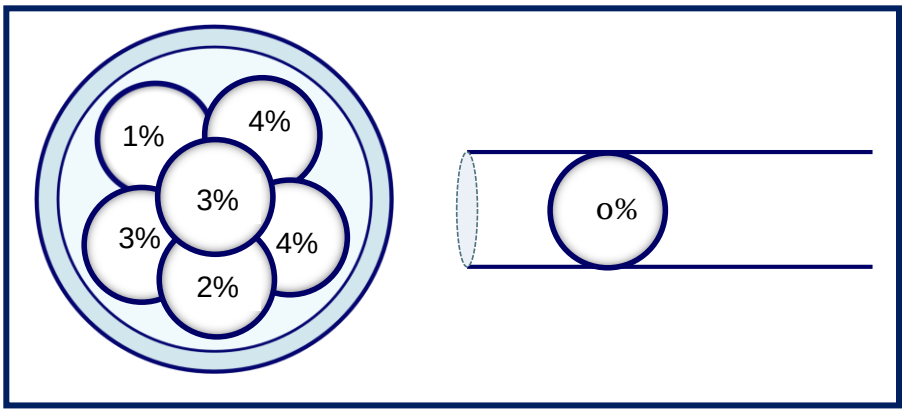


Figure3. 9 Schematic diagrams represent mutation load analysis performed upon individual blastomeres disaggregated from the same embryo both for the mt.9176T>C.

Above schematic diagram represents two cleavage stage embryos which arrested development and were able to be manually disaggregate in order to obtain individual blastomeres (a) n=5 and (b) n=6 for the m.9176T>C mutation. The mutation load was determined for the individual blastomeres with mutation loads ranging from 1-4% (mean=3%) and 2-4% (mean=3%) respectively. These two embryos had previously been biopsied on day 3 as part of the PGD programme (ran between the Newcastle upon Tyne NHS fertility centre at Life and the NCG mitochondrial diagnostic service). Here no mutation was detected in either of the blastomeres analysed (analysis performed by Mrs C. Alston).

3.4.3 Mutation load analysis conducted for embryonic samples obtained from a carrier of the m.8993T>C mutation

3.4.3.1 Studies examining mtDNA mutation loads in embryos

Several samples were obtained from a patient known to be a carrier of the m.8993T>C mutation. The patient had been found to carry the mutation load at 96% and 98% in blood and urine samples respectively. Due to carrying the mutation at such high levels the couple opted to undergo PGD. Several embryos were created following IVF treatment (n=4) and biopsied on day 3. Unfortunately all biopsies blastomeres were found to possess the mutation to virtual homoplasmic levels (99%, 98%, 99% and 99% respectively) (Mutation loads for biopsied blastomeres analysed by Mrs C.Alston, clinical scientist at the NCG mitochondrial diagnostic service). Later mutation loads were analysed for the whole embryos, which were previously biopsied (99%, 98% and 99% respectively) (Table3.1) (Fig3.10a). Again mutation loads observed in the embryos were reflective of mutation loads observed in the earlier blastomere biopsied samples, demonstrating the accurate value of biopsied cell (It should be noted that the fourth embryo failed to amplify successfully therefore was not included in analyses).

3.4.4 Mutation load analysis conducted for embryonic samples obtained from a carrier of the m.10158T>C mutation

3.4.4.1 Studies examining mtDNA mutation loads in embryos

Mutation loads were determined for n=2 embryos unable to be disaggregated into individual blastomeres and therefore analysed as whole embryos. Here mutation loads determined for the whole embryos (18% and 4%) were similar to those levels previously determined for the biopsied blastomeres (18% and 0%) (Fig3.10b) (mutation loads determined by Mrs C.Alston for biopsied blastomere).

3.4.4.2 Studies examining mtDNA mutation loads for polar body biopsied oocytes

A single *in vitro* matured MII stage oocyte was biopsied allowing mutation loads to be determined for the oocyte (1%) and polar body (1%) separately (Fig3.8c). The corresponding values observed between the polar body and its corresponding oocyte were identical demonstrating the predictable value of the polar body.

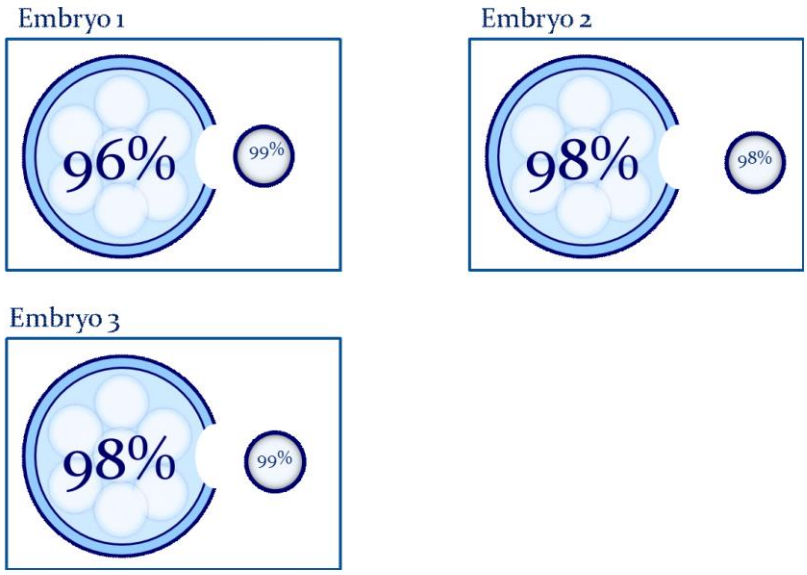
mt.8993T>C	Biopsied Blastomere mutant load	Remaining embryo mutant load
Embryo 1	99%	96%
Embryo 2	98%	98%
Embryo 3	99%	98%

Table3. 1Mutation loads determined in individual blastomeres for the 8993T>C mutation.

Mutation loads were determined for a total of n=3 whole embryos which were had been biopsied on day 3 as part of the PGD programme(run between the Newcastle upon Tyne NHS Fertility Centre at Life and the NCG mitochondrial diagnostic service) and the mutation loads determined for the biopsied single blastomeres (99%, 98% and 99%). Unfortunately these embryos contained a high degree of fragmentation and therefore were unable to be disaggregated in order to obtain individual blastomeres, consequently all three embryos were analysed as whole embryos (96%, 98% and 98%).

m.8993T>C

a



m.10158T>C

b

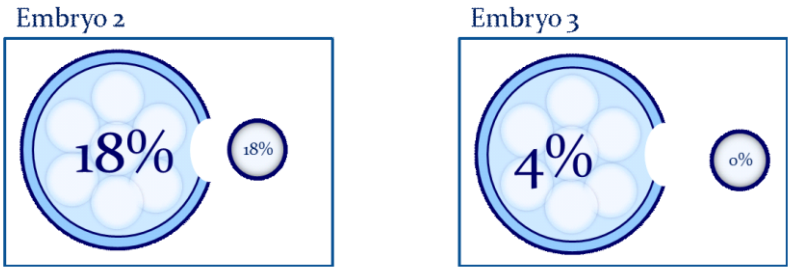


Figure3. 10 Mutation load analysis for whole embryos for the m.8993T>C and m.10158T>C mutation

Here we were able to analyse mutation loads in a total of three whole embryos which were unable to be disaggregated into individual blastomeres due to the high degree of fragmentation. **(A)** Mutation loads were determined for those embryos analysed for the m.8993T>C mutation (96%, 98% and 98%). All three embryos had previously been biopsied on day three and a single blastomere obtained for mutation load analysis via pyrosequencing (99%, 98% and 99%). **(B)** Mutation loads were determined for two embryos for the m.10158T>C mutation (18% and4 %). Both embryos had previously been biopsied on day three and a single blastomere obtained for mutation load analysis via pyrosequencing (18% and 0%).

mtDNA mutation	Oocyte Sample identification Number	Heteroplasmy (%)
m.3688G>A	GV oocyte	13
	MI (zona free oocyte)	3
	MII (with large polar body)	4
m.14709T>C	GV oocyte	4
	MI oocyte	28
	MII oocyte	31

Table3. 2 Mutation loads determined for oocytes sampled for the m.3688G>A and m.14709T>C mutations

Mutation loads were determined for a total a number of oocytes (n=6) for both the m.3688G>A (n==3) and m.14709T>c (n=3) mutations, each at various stages of maturity. Mutation loads for those oocytes from the carrier of the m.3688G>A mutation were determined as: germinal vesicle(GV)stage oocyte 9%, Metaphase I (MI) stage oocyte 3% and Metaphase II (MII) stage oocyte 4%. This was repeated for those oocytes obtained from the carrier of the m.14709T>C, here these were determined as: Germinal vesicle (GV) stage oocyte 4%, Metaphase I (MI) stage oocyte 28% and Metaphase II (MII) stage oocyte 31%.

3.4.5 Mutation load analysis conducted for embryonic samples obtained from a carrier of the m.14487T>C mutation

3.4.5.1 Studies examining mtDNA mutation loads in embryos

A number of embryos (n=8) cryopreserved at the eight cell stage were received from a patient undergoing fertility treatment at an IVF centre in Canada (Hewko, Calgary, Canada). All embryos had been frozen at the 8-cell stage and were warmed at different points using medicult thawing solutions and protocol (as fully described in material and methods section). An attempt was made to biopsy the first thawed embryo following appropriate recovery. This resulted in the successful retrieval of one single blastomere (9%) which was analyzed together with the remaining fragmented embryo (7%). Three further embryos were thawed and disaggregated manually to obtain individual blastomeres (n=34). The heteroplasmy levels were low in all blastomeres analysed and ranged from 1 to 11% (mean ~4%) overall (Table3.3) (Fig3.11).

3.4.5.2 Studies examining mutation loads in blastocyst stage embryos

The remaining four embryos thawed were cultured and developed to blastocysts (Figure3.2). An attempt was made to separate the inner cell mass (ICM) from the trophectoderm for those blastocysts with clearly visible ICM (n=3). The mutation levels determined for the trophectoderm samples (17%, 2% and 4%) correlated with levels found in the ICM (12%, 3% and 3%) for all three embryos analysed in this way (Fig3.12a, b and c). In so revealing no preferential segregation of mtDNA species between the two cell lineages at least in samples analysed for these two particular mutations. It was not possible to isolate the ICM cells from the remaining trophectoderm cells for one of the thawed embryos which developed to a blastocyst therefore the mutation load was determined for the entirety of the blastocyst (ICM + trophectoderm); this was determined to be 20% (Fig3.12d).

Samples which we analysed for this particular mutation were from a lady who had an affected family member and wanted to determine her risk of having an affected child.

We were only able to detect very low levels of the mutation (>10%) within individual blastomeres, whole embryos and blastocyst stage embryos which would all have been within minimal risk. This would fit with previous reports in the literature where variable levels of heteroplasmy have been detected between affected and unaffected members of the same family with only those with very high levels of heteroplasmy actually displaying clinical symptoms (Wang *et al*, 2009).

m. 14487T>C Embryo	Fragmented Embryo	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Average mutation load
Embryo 2	7	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	~8%
Embryo 3	2	5	2	2	5	6	-	-	-	-									~4%
Embryo 4	2	5	3	4	1	2	3	2	6	6	6	5	6	2	4	4	11	3	~4%
Embryo 5	4	5	2	7	7	4	3	2	2	4	2	4	-	-	-	-	-	-	~4%

Table3. 3 Mutation loads determined in individual blastomeres by pyrosequencing analysis for the 14487T>C mutation.

Here individual blastomeres were obtained for, four frozen and thawed embryos which were obtained for analysis for the m.14487T>C mutation. Here many of the embryos contained few intact cells due to an artefact of the freeze thaw process. However attempts were made to salvage individual blastomeres from all embryos (n=4). Blastomeres were able to be retrieved and subsequently mtDNA mutation loads analysed for each. For embryo 2, only one intact blastomere was able to be analysed (n=1, 9%) The mutation load was analysed in several blastomeres for embryo 3 (n=5) ranging from 2-6% (mean=4%) A total of n=17 blastomeres were obtained for embryo 4, mutation loads ranging from 1-11% (mean=4%) and finally n=11 blastomeres were obtained for embryo 4 with mutation loads ranging from 2-7% (mean- =4%).

m.14487T>C

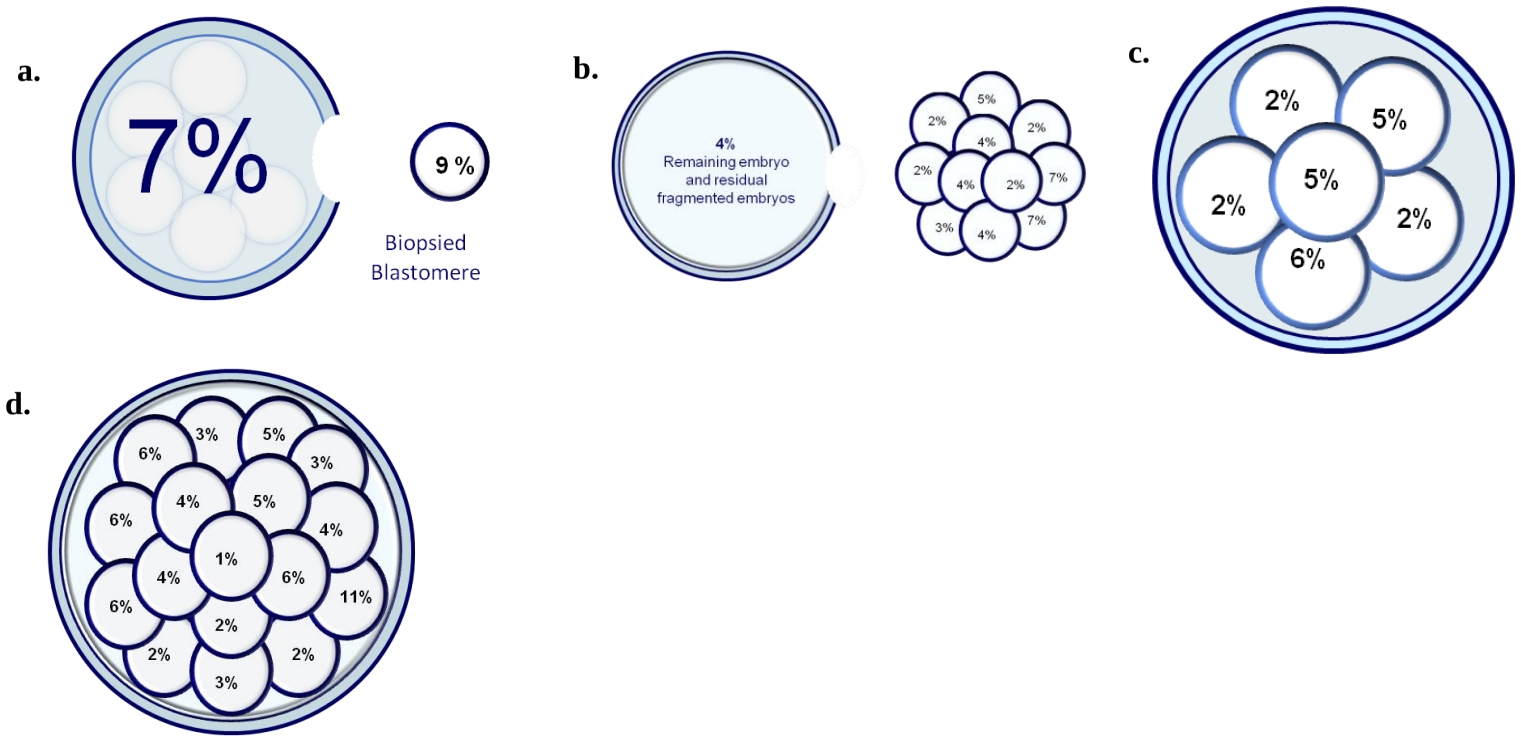


Figure3. 11 Schematic diagrams represent mutation loads determined for a number of various stage (cell-number) cleavage stage embryos for a carrier of the m.14487T>C mutation.

Here embryos were frozen by conventional slow freezing at the 8-cell stage and later thawed cultured and where possible disaggregated into individual blastomeres for mtDNA mutation load analysis. (a) Embryo contained substantial fragmentation and whole embryo was collected for analysis, mutant load determined as 7%. (b) Here a total of 11 blastomeres were able to be disaggregated and mutation load analysed for each of the cells (average mutant load across the embryo ~4%). (c) Total of 6 blastomeres were obtained for embryo three (~ mutant load) (d) Total of 17 blastomeres were obtained for embryo 4

m.14487T>C

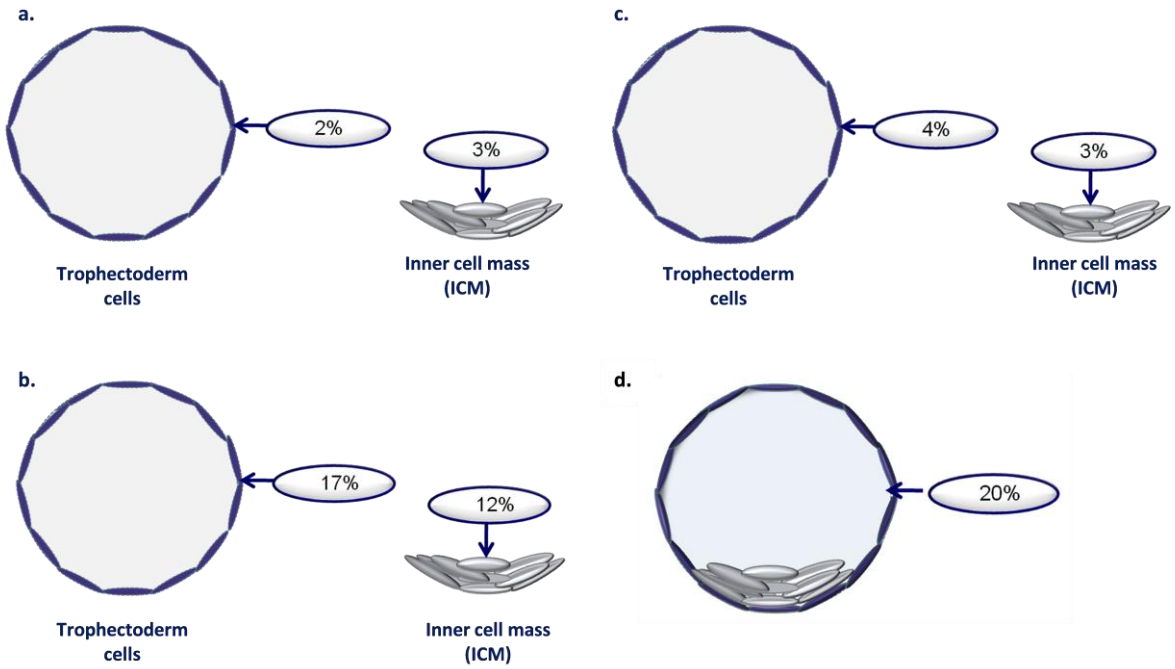


Figure3. 12 Determination of mutation loads in pre-implantation blastocysts for the m.14487T>C mutation.

Here mutation loads were determined for three blastocysts which were able to be dissected into respective inner cell mass (ICM) and trophectoderm cells following thawing (a, b and c). However the inner cell mass was manually dissected from one of the embryos which developed to a blastocyst was used to attempt stem cell derivation and therefore it was therefore it was only possible to perform mtDNA mutation load analysis on the remaining trophectoderm and residual inner cell mass,(20%) (d). unfortunately, no additional mutation load data was available for these this blastocyst

3.4.6 Mutation load analysis conducted for embryonic samples obtained from a carrier of the m.14709T>C mutation

3.4.6.1 Studies examining mtDNA mutation loads in embryos

Several samples were received from a patient undergoing fertility treatment at the Newcastle upon Tyne NHS Fertility centre at life. These embryos (n=3) had been biopsied on day 3 by Dr Lyndsey Butterworth. One arrested development at the 8-9 cell-stage and individual blastomeres were obtained by manual disaggregation (n=8, including 2 blastomeres that could not be disaggregated into individual blastomeres and were therefore analysed together), a maximum intra-blastomere variation of 7% was observed with mutation loads ranging from 41-48%, (mean=45%). The mutation load determined for the biopsied cell was 45%, which correlated with loads detected in the disaggregated blastomeres (Fig3.13).

3.4.6.2 Studies examining mutation loads in blastocyst stage embryos

The two other biopsied embryos successfully developed to blastocyst stage embryos therefore here it was possible to determine mutation loads were for combined trophectoderm and *zona pellucida* samples (collected day 7) Mutation loads detected in the biopsied blastomeres (34 and 39%) for these two embryos which developed to blastocysts were reflective of those detected in the trophectoderm and *zona pellucida,* (33 and 31% respectively). Interestingly mutation loads observed within the individual blastomeres and later stage blastocyst samples were similar to loads previously observed in samples analysed for the female carrier of the mutation when both skeletal and urine (40 and 49%)(Fig3.15).

3.4.6.3 Studies examining mtDNA mutation loads in oocytes

Mutation loads were also determined for several unfertilised oocytes obtained from the carrier of the m.14709T>C mutation at various stages of maturation (n=3) here the mutation loads were determined as: 4% in a GV stage oocyte, 28% in a metaphase I

stage oocyte and 33% in a metaphase II stage oocyte (Fiigure3.14). Much greater variation was observed between the oocytes we analysed in comparison to the blastomeres we analysed for the same mutation.

m. 14709T>C

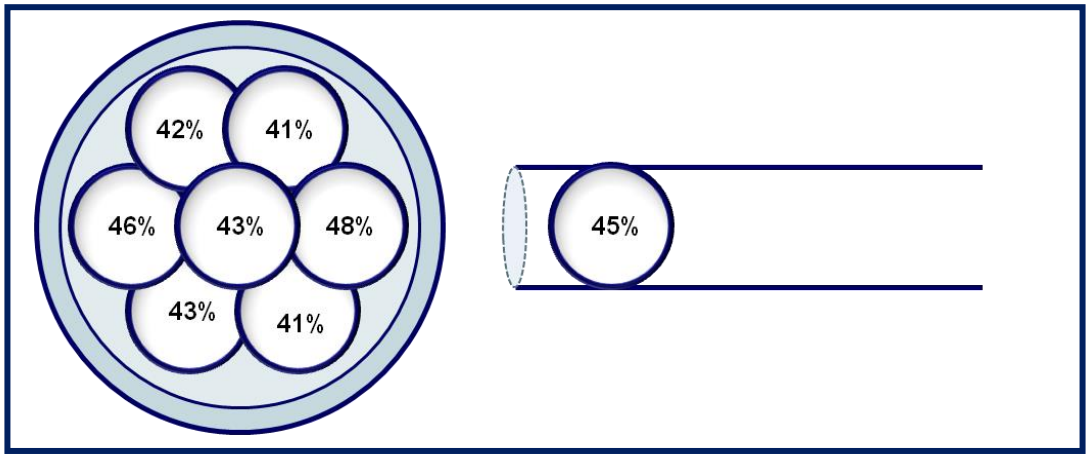


Figure3. 13 Schematic diagrams represent mutation load analysis performed upon individual blastomeres disaggregated from the same embryo for the m.14709T>C mutations

Above schematic diagram represents a cleavage stage embryo obtained from a carrier of the m.14709T>C mutation. Following manual disaggregation (n=8, including 2 blastomeres that could not be disaggregated into individual blastomeres and were therefore analysed together), a maximum intra-blastomere variation of 7% was observed with mutation loads ranging from 41-48%, (mean=45%). This embryo had previously been biopsied on day 3 as part of the PGD programme (run between the Newcastle upon Tyne NHS Fertility Centre at Life and the NCG mitochondrial diagnostic service). Here the mutation load had been determined as 45% (analysis performed by Mrs C. Alston).

m. 14709T>C

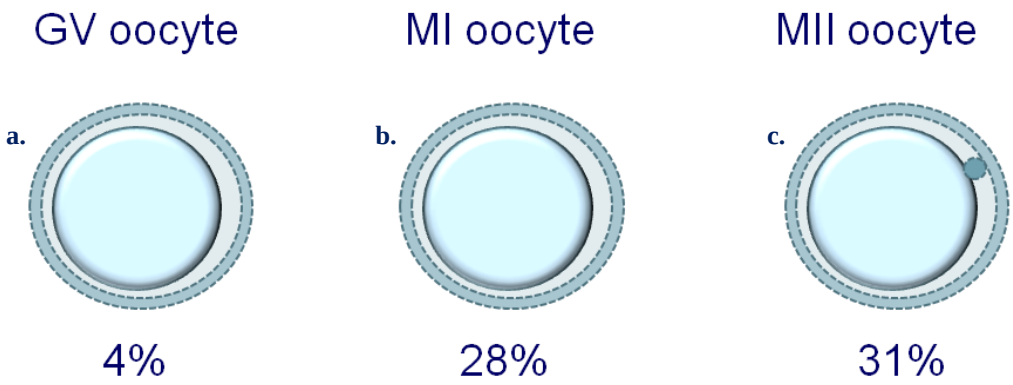


Figure3. 14 Schematic diagrams represent mutation load determined in oocytes sampled from a carrier of the m.14709T>C mtDNA mutation.

Here a number of oocytes (n=3) at various stages of maturity were obtained from a carrier of the m.14709T>C mutation. Mutation loads were subsequently determined for each (a) Germinal vesicle (GV) stage oocyte (4%). (b) Metaphase I (MI) stage oocyte (MI) (28%) and (c) Metaphase II (MII) stage oocyte (31%).

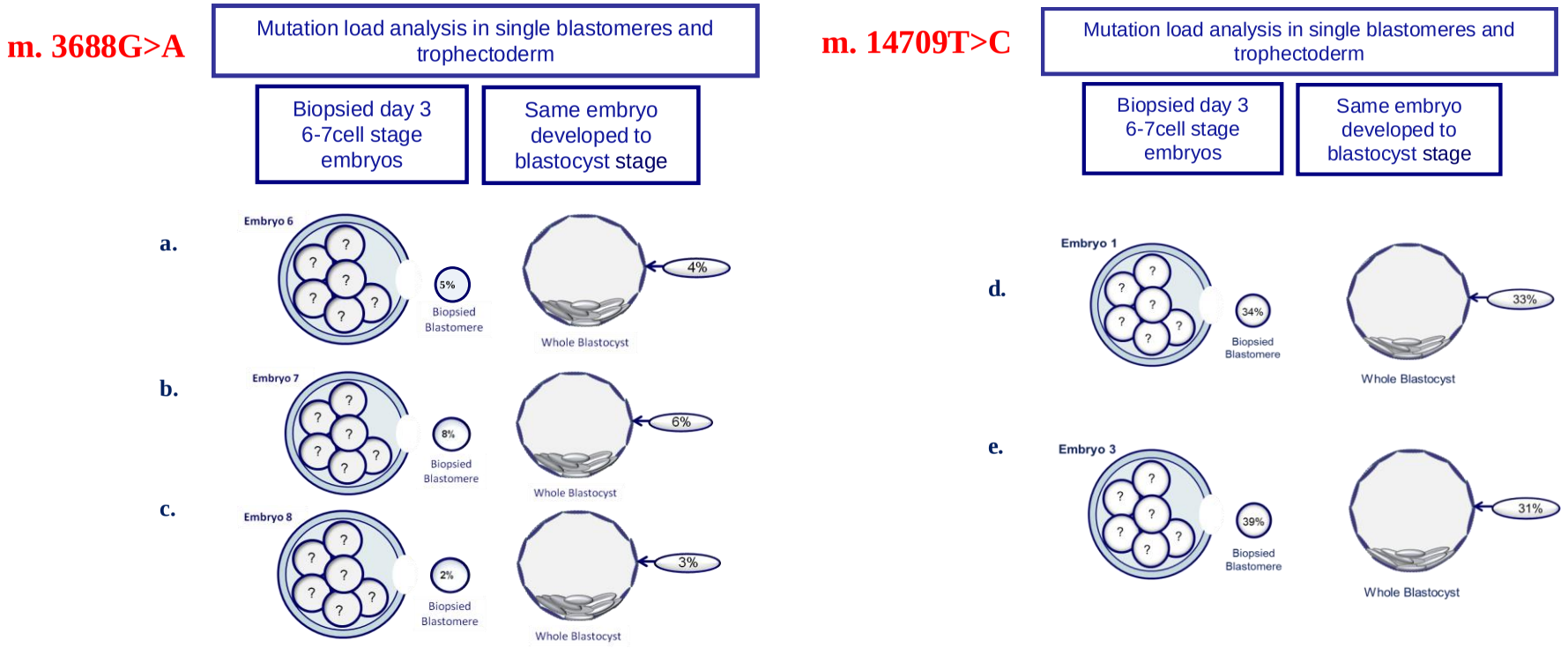


Figure3. 15 Schematic diagrams represent mutation loads determined in samples obtained from a carrier of the m.3688G>A and m.14709T>C mtDNA mutations.

Embryos were biopsied at day 3 and left in culture to develop, subsequently two embryos developed to blastocysts and the trophoectoderm cells and residual inner cell mass were collected for mtDNA mutant load analysis. (a) Biopsied blastomere 16% corresponding mutant load in trophectoderm cells 4%. (b) Mutant load determine for biopsied blastomere as 8% and corresponding trophectoderm cells as 6%, (c) biopsied blastomere 2% corresponding mutant load in trophectoderm cells 3%. (d) Biopsied blastomere 34% corresponding mutant load in trophectoderm cells 33%. (e) Mutant load determine for biopsied blastomere as 39% and corresponding trophectoderm cells as 31%.

3.5 Discussion

The lack of available treatment for mitochondrial disease means that many families with inherited mtDNA disease are left with difficult reproductive choices. Until recently these choices were limited to genetic counselling, ovum donation and chorionic villus biopsy/amniocentesis. The work of Steffann et al, have shown that PGD is now feasible for some families with mtDNA disease, where the high copy number of mtDNA makes the analysis less prone to artefacts like amplification failure and allelic-drop-out (Thorburn, 2004). Although the number of children born following PGD for mtDNA disorders remains limited and the data regarding the distribution of mutation loads within embryos minimal and available for very few mtDNA mutations. Therefore the data we have gathered has allowed us to gain an insight into mtDNA distribution within oocytes and pre-implantation embryos for several different mtDNA point mutations. This provided a unique opportunity to broaden our current understanding regarding mtDNA segregation, during these critical early pre-implantation stages of development.

Here I employed pyrosequencing technology to analyze mtDNA mutation loads in oocytes, single blastomeres, pre-implantation and blastocyst stage embryos received from a total of seven patients undergoing pre-implantation genetic diagnosis at the Newcastle upon Tyne NHS trust fertility Centre at Life. For samples which were not able to be included as part of their treatment cycles (refer to materials and methods section for further sample collection details).

The analysis of individual blastomeres obtained for four different mtDNA mutations (m.3688G>A, m.9176T>C, m.14487 and m.14709T>C) demonstrated the even distribution of mutation loads between blastomeres originating from the same embryo. Minimum variation was observed between blastomeres, ranging from as low as 1-10% maximum (Table3.5). This is in-line with previous studies which have examined mutation loads in individual blastomeres obtained from human embryos for the m.8993T>G and m.3243A>G mutation who reported

observing limited variation ranging from 2-11% and 0.5% to 2.9% (Tajima et al, 2007; Treff et al, 2012). Our observations are therefore entirely compatible with the even spread of mutation previously reported (Steffann et al, 2006; Tajima et al., 2007; Treff et al, 2012). This is important as previously it had been suggested that mutation loads observed in biopsied cells may not portray an accurate representation of the mutation load in the remaining cells of the embryo (Bredenoord et al, 2008) due to the unpredictable manner in which mitochondria are inherited and segregate making screening procedures such as pre-implantation genetic diagnosis (PGD) impossible to perform. However my data suggests this is not the case. As those mutation loads I observed in both the individual blastomeres and whole embryos both agreed with mutation loads determined for the biopsied blastomeres obtained on day 3 as part of the PGD programme (Table3.5). This is in agreement with previous reports made for two different mutations (m.8993T>G and m.3243A>G) (Gigarel et al2011; Monnot et al, 2010; Steffann et al, 2006; Treff et al, 2012). It would appear that mutation loads observed within biopsied blastomeres provides an accurate representation of the mutation loads within the remaining cells of the embryo, providing much needed evidence in support of PGD for mtDNA inherited disorders.

Although it is important to ensure that the mutation load observed for the biopsied cell on day 3 accurately reflects the mutation load in the remaining cells of the embryo. It is also equally important that we are certain that the mutation load observed following biopsy is not subject to not change during the pre-implantation period. This type of analysis is often difficult to perform in clinical practice, as non-essential biopsy is often avoided due to the negative effect this can exert on future developmental potential. However after obtaining a number of samples obtained from patients undergoing fertility treatment or PGD at the Newcastle Fertility Centre at Life, which were unsuitable for use in treatment or cryopreservation this material was made available for mutation load analysis and so making such analyses possible to perform. A total of 5 embryos were able to be included in this particular analysis, which were obtained from two patients each a carrier of a different mtDNA mutation, including the m.3688A>G (n=3) and m.14709T>C (n=2). A high degree of similarity was observed between the biopsied blastomere (obtained on day 3) and the pre-implantation trophectoderm

(obtained between day 5-7) for samples obtained for both mutations. These studies support the work from Monnot et al (2011) who showed similar levels of the m.3243A>G mutation in individual day 3 blastomeres and corresponding day 5 trophectoderm samples. This suggests that mtDNA segregation is not skewed across embryonic and extra-embryonic cells at least at this developmental stage (Monnot et al, 2011).

However the trophectoderm only represents those cells which will give rise to the placental tissues, whose role it is to support the embryo during uterine development. Instead it is the cells which comprise the 'inner cells mass' (ICM) which go onto give rise to the 'embryo proper'. Therefore ensuring that the mutation load present in these cells would more likely provide more useful information as to the levels of mtDNA we might expect to see in the resulting child at birth. Therefore more recently efforts have been made to examine mutation loads in blastocyst stage embryos obtained from carriers of mtDNA mutations and through the use of micromanipulation techniques, manually separate the 'trophectoderm cells' from those cells which comprise the 'ICM'. This particular form of analysis allows to see how accurately representative mutation loads observed in the trophectoderm are of the actual mutation load found within the cells of the 'ICM'. As part of this study I was able to perform such analyses, which revealed that the mutation load observed in the trophectoderm was indeed highly comparable with the mutation loads observed for the cells of the 'ICM' for two different mtDNA mutations (m.14487T>C and m.3688G>A), suggesting that mutant mtDNA is uniformly segregated between all cells of the premiplantation embryo up to the blastocyst stage. This is supported by results obtained from embryos carrying the m.3243A>G mutation, which showed a maximum variation of 9% mutant load between the ICM and TE (Treff et al, 2012). In addition, multiple biopsies from the ICM and TE of the same embryo revealed highly consistent heteroplasmy levels across the embryo, with mutation loads differing by 2-4% in the ICM and 3-11% in the TE (Treff et al, 2013). The low variation within the ICM supports the observation that mutant load remains relatively stable across fetal tissues (Matthews et al, 1994, Ferlin et al, 1997, Cardailoi et al, 2000, Monnot et al, 2010) and suggests that the mutant

load determined at the time of biopsy will remain unchanged during embryo-fetal development.

	Biopsied Blastomere	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	Average mutation load
m.14709T>C Embryo (2)	45	42	41	46	43	48	43	41	-	-	-	-	-	~43%
m.3688G>A Embryo (4)	4	5	6	6	2	1	4	5	4	7	5	5	5	~5%
m.9176T>C Embryo (2)	0	3	4	3	2	4	-	-	-	-	-	-	-	~3%
m.9176T>C Embryo (3)	0	1	4	3	4	3	2	-	-	-	-	-	-	~3%

Table3. 4Mutation loads determined in individual blastomeres by pyrosequencing for several different mtDNA point mutations

Here embryos were biopsied day 3 (as part of the PGD programme) and mutation loads determined (by the NCG mitochondrial diagnostic service). Embryos were then disaggregated and individual blastomeres obtained for each of the embryos. (a) m. 14709T>C mutation load determined for biopsied cell (45%) and corresponding blastomeres (41-48%) (b) m. 3688G>A – mutation load for biopsied cell (4%) and corresponding blastomeres (1-7%) (c) mt.9176T>C mutation – cells biopsied for both embryos were both determined as mutation free (0%) corresponding blastomeres for both embryos both contained minimal levels of the mutation (1-4%).

		Heteroplasmy (%)			
mtDNA mutation	**Sample Identification Number**	**Biopsied Blastomere**	**Whole Blastocyst**	**Inner cell mass**	**Trophectoderm**
m.14709T>C	**Embryo 1**	39	-	-	31
	Embryo 3	34	-	-	33
m.14487T>C	**Embryo 1**	-	-	-	20
	Embryo 6	-	-	12	17
	Embryo 7	-	-	3	2
	Embryo 8	-	-	3	4
m.3688G>A	**Embryo (1)**	-	-	2	2
	Embryo (2)	-	3	-	-
	Embryo (6)	5	4	-	-
	Embryo (7)	8	6	-	-
	Embryo (8)	2	3	-	-

Table3. 5Determination of mutation loads in biopsied blastomeres and their correlation to corresponding pre-implantation samples

Here several embryos were biopsied on day 3 (post-fertilisation) and then cultured all three developed to blastocysts and were subsequently collected for mtDNA mutation load analysis**.** Mutation loads were also determined for a number of blastocysts (d and e) which were biopsied at day three however blastomeres failed to amplify. A number of blastocysts (e) were able to be separated into trophectoderm and inner cell mass, which were both able to be analysed separately to determine if there are any differences in mutation load segregation between the two cell lineages.

Indeed trophectoderm biopsy is now increasingly being viewed as a preferred in favour of the traditional approach of cleavage stage biopsy (Dokras et al, 1990; Montag et al, 1998; De Boer, et al, 2004). This is because it offers several important benefits. Firstly it an increased number of cells are able to be analysed from a trophectoderm biopsy (2-5 cells v 1cell). Secondly it is often regarded as a safer procedure, as it avoids removing cells which could contribute to the embryo proper. Many argue that it is much safer to remove a small number of cells from a blastocyst which can contain up to 300 cells, in comparison to an 8-cell cleavage stage embryo. Thirdly embryos which attain development to the blastocyst stage, have demonstrated developmental potential and therefore this increases the chances of a successful transfer (De Boer et al, 2004; Braude et al, 2002). Therefore if mutation loads observed in the trophectoderm is representative of mutation levels observed in the ICM then blastocyst stage biopsy should not be discouraged for mtDNA disease PGD patients. However there is a disadvantage to performing trophectoderm biopsy for PGD, due to the time required for molecular analysis to be performed before samples can be selected for transfer. As the molecular analysis required to assess mutation loads often takes between 24-48 hours, blastocysts must be able to be cryopreserved, until the required mutation load information has been obtained. Vitrification at the blastocyst stage is possible, although it is imperative that an efficient vitrification programme is put in place before commencing a trophectoderm biopsy PGD programme.

Following the 74th ENMC meeting a set of criteria were agreed which a patient should meet prior to consideration for PND or PGD, this includes: a close correlation between the mutant load and disease severity, a uniform distribution of mutant load across tissues and no major change in mutant load with time. By meeting these criteria it was hoped that successful outcomes would be achievable for patients opting to undergo PND and PGD. In our recent study we found that levels observed in the female carriers we had access to samples for (m.3688G>A, m.10158T>C and m.14709T>C) reflected the mutation loads detected in her embryonic samples, and in some instances (m.3688G>A) to a much lesser or even undetectable levels. This allowed us not only to determine the efficiency of the pyrosequencing assays used to quantify levels of heteroplasmy in the samples but also allowed us to determine the relationship with levels observed in her embryonic samples. This is important as the complex genetic and phenotypic variability characteristic of mtDNA inherited disorders has previously

compounded attempts to identify potential relationships between the levels of mutant mtDNA observed in the mother and the clinical features likely to be expected in her offspring (Chinnery et al, 1998). Indeed a number of studies have attempted to obtain a more accurate description of the inheritance of pathogenic mtDNA mutations through the study of large pedigrees (Chinnery et al, 1998). However the majority of these studies conducted have cautioned that by no means do the conclusions reached provide absolute given risks regarding the likelihood of them conceiving a child with a given clinical phenotype. Instead the outcome of pregnancy is likely related to the level of mutant mtDNA observed in the mother under discussion and that the risks of having affected offspring may differ between mtDNA mutations.

3.5.1 Preconception genetic diagnosis (PCD) for mtDNA inherited disorders

Preconception genetic diagnosis (PCD) is now routinely performed for a number of nuclear encoded genetic diseases. Here the first or second polar body is biopsied and the mutation load analyzed without altering the integrity of the oocyte of embryo. It is increasingly being employed in countries where cleavage-stage embryo selection is legally not possible to perform and ever more sought after by couples whom are concerned by potential embryo wastage issues associated with conventional cleavage stage biopsy (Gigarel et al, 2011). However two recent publications reported rather conflicting data regarding the predictive value of the polar body in relation to the oocyte (Gigarel et al, 2011; Vandewoestyne et al, 2011). The study published by Vandewoestyne et al (2011) reported observing a strong correlation in levels detected between oocytes and their respective polar bodies for the m3243A>G mutation. This was in contrast to the study results published by Gigarel et al (2011) who demonstrated the lack of predictive value the polar body represented in relation to the oocyte following examination of mutation loads in 50 polar bodies and their corresponding oocytes. Indeed this same study was further able to demonstrate that this discrepancy they observed between mutation loads observed in the polar body and corresponding oocyte was more apparent at high mutation loads (>60%) This could be due to the lower amount of mtDNA found within polar bodies which could make analysis susceptible to allelic drop-out or indeed preferential amplification. This therefore questioned the potential effectiveness of PCD for mitochondrial disease however it does not fully discount its potential to select ‘mutation free’ oocytes, in so reducing embryo

wastage. However our own recent examination of mutation loads in oocytes and their corresponding biopsied polar bodies, for the two mutations: mt.3688G>A and mt.10158T>C revealed a high correlation between the mutation load observed between the polar body and its corresponding oocyte. Again however it must emphasized that the mutation loads we observed within these samples was relatively low in comparison to the spectrum of mutation loads samples contained in the study reported by Gigarel e et al (2011). This study state that the correlation which was present between the two samples was indeed lost when a threshold value of >60% was observed within the oocyte. This may indeed to the difference in mtDNA copy number between the oocyte body and the polar body, which results in a loss of predictive power by the biopsied polar body.

3.5.2 Studies of mutation loads in oocytes from carriers of mtDNA mutations

Several studies have now examined mutation loads in oocytes obtained from carriers of mtDNA point mutations (Blok et al, 1997; Dahl et al, 2000; Brown et al, 2001: Jacobs et al, 2007; Monnot et al, 2011). The variation in mutation loads we have observed amongst the oocytes analysed thus far for both the m.3688G>A and m.14709T>C mutation is in-line with previously published data (Brown et al, 2001; Jacobs et al, 2007), which demonstrate the vast degree variance in mutation loads amongst the oocyte population.

Indeed a study performed just over a decade ago (Brown et al, 2001) looked to examine mutation loads in several primary oocytes (~82) obtained from a woman who harboured the m.3243A>G (MELAS) mutation. The woman was asymptomatic but known to carry the mutation at varying levels (18.1% in quadriceps and 7.24% in leukocytes). The proportion of mutant mtDNA found within her oocytes ranged from 0% to 45% (mean 12.64%). This study inferred that random genetic drift was likely to be responsible in this instance for determining the level of mutant mtDNA observed within primary oocytes. A similar study recently reported examination of mutation loads in oocytes for a carrier of the MELAS (m/3243A>G) mutation with mutation loads ranging from 9% to 90% (Treff et al, 2012). Others have reported observing between <5% to >50% variation in mutation loads between oocytes (Jacobs et al, 2007). Indeed

I also observed variation in mutation loads for those oocytes I analysed for both the m.3688A>G (3% to 13%) and m/14709T>C mutation (4% to 31%).

As this study was only able to analyse mutation loads for a small number of oocytes due to the scarcity and availability of this material, it is encouraging that other groups have also been able to demonstrate the similar variability in mutation loads within the oocyte population of mtDNA mutation carriers as this substantiates the data presented here within this thesis. Taking all current data into account it would therefore appear that the oocyte population does harbour the greatest degree of variability in mtDNA mutation loads. This is most likely due to random genetic drift during germ cell development. It is not possible as yet to say if there is some underlying mechanism which may influence which oocytes are likely to possess best fertilization potential and if indeed those oocytes carrying the mutation possess a selective advantage against those which do not and if indeed this is in some way mutation specific. However the variability observed within the oocyte population may reflect the variability observed between siblings born from mitochondrial DNA mutation carriers.

3.6 Conclusion

Our results presented here support similar observations made by other studies for different mutations whereby the level of mutation is very evenly spread throughout the embryo and thus is supportive of both antenatal and pre-implantation diagnosis as valuable approaches for families with different heteroplasmic point mtDNA mutations. However whether PGD is suitable for all heteroplasmic mtDNA mutations remains to be seen but it is essential that we continue to collect information on additional mtDNA mutations so that we can provide best possible advice to women exploring the different reproductive options.

Chapter 4

Identifying key factors involved in successful Pronuclear Transfer (PNT)

Chapter 4 Identifying key factors involved in successful Pronuclear Transfer (PNT)

4.1 Introduction

Mitochondrial disease is now recognised as a common cause of genetic disease (Schaefer et al, 2004). Current estimates show a minimum of 1 in 10,000 adults in the UK are affected by mtDNA disease (Schaefer et al, 2008), with 1 in 6,000 women in the UK heteroplasmic for a particular mtDNA mutation (Schaefer et al. 2008). At present there are no successful treatments for mitochondrial disease and current strategies are predominately targeted at minimising symptoms. Due to the lack of available treatment for these patients and their families, preventing the transmission of mitochondrial disease is a priority (Chinnery et al, 2006; Craven et al, 2010).

One strategy currently being explored with the aim of preventing transmission of mitochondrial disease is the nuclear transfer technique, pronuclear transfer (PNT). Studies conducted in the lab have successfully demonstrated that the transfer of pronuclei between abnormally fertilised human embryos results in minimal carry-over of donor zygote mtDNA and is importantly compatible with onward development (Craven et al, 2010).

The efficiency and feasibility of PNT to prevent transmission of mtDNA disease has been successfully demonstrated using the abnormally fertilised human zygotes. However it is recognised that these abnormally fertilised zygotes have a limited potential for development therefore it will be essential to perform these same experiments in normally fertilised human zygote, which are known to contain only two pronuclei. This it is hoped will allow for improved potential for onward development to the blastocyst stage, further demonstrating the potential of the technique. However just as these experiments will have to be performed in normally fertilised human zygotes it is highly likely that other operators, will have to be trained to perform the procedure to demonstrate. However first it will be important to establish that the technique can be reproduced with similar results. Therefore the purpose of this chapter was to establish important factors which are key to performing the PNT procedure

successfully and efficiently and also determine if polar body removal may improve outcomes.

4.2 Aims:

- Establish factors involved in successful pronuclear transfer manipulations.
- Monitor onward development following pronuclear transfer and contrast with previously gathered published data.
- Maximise development of post-manipulated embryos
- Examining the potential and feasibility of polar body removal

4.3 Materials and Methods

4.3.1 Collection of Human Oocytes and Embryos

In this study all embryos were obtained from patients undergoing routine fertility treatment at the Newcastle Fertility Centre at Life, following informed consent. The project had previously been approved by the Human Fertilisation and Embryology Authority (HFEA).

4.3.2 Abnormally Fertilised Human Embryos

Embryos used in this study included abnormally fertilised human embryos collected on day 1 of the IVF or ICSI cycle. These embryos were identified as abnormal by the presence of either 1 pronucleus (mono-pronucleate) or 3 pronuclei (tri-pronucleate) 18-19 hours post insemination. These embryos were unable to be used for fertility treatment and are routinely rejected from the IVF programme.

4.3.3 Embryo Manipulations performed between abnormally fertilised PN stage embryos

Embryos were transferred to a manipulating dish containing 4µl sized drops of G1v5 plus media (vitrolife AB, Kungsbacka, Sweden) supplemented with 0.5µl of

cytochalasin B (1µg/ml) and 1µl nocodazole (2µg/ml) (Sigma-Aldrich, UK) Embryos were left to equilibrate in the manipulation media for between 15-30 minutes prior to manipulation. Manipulations were subsequently performed using an inverted microscope (Nikon Eclipse TE2000-U) equipped with a micromanipulation system (Integra Ti, Research Instruments). Both holding and biopsy pipettes were lowered into a drop in the manipulating dish not containing an embryo and both pipettes primed before the embryo was immobilised by gentle suction with the holding pipette (Figure4.1a). Subsequently all manipulations were carried out at the x40 magnification, the objective to which the laser was fitted. The embryo was moved towards the laser target (XYclone laser, clonetech) and a small hole was created in the *zona pellucida*. Using the biopsy pipette pronuclei were gently removed from the embryo, after which embryo was released from holding pipette (Figure 4.1b, c and d).

4.3.4 Embryo enucleation and karyoplast transfer

Following successful removal of the PN karyoplast, the biopsy pipette containing the PN karyoplast (s) was taken to the HVJ-E (GenomONETM-CFEX HVJ Envelope Cell Fusion Kit, Cosmo Bio Co) drop. The PN karyoplasts were then expelled into the drop of HVJ-E to coat the karyoplast in a sufficient amount of HVJ-E. The PN karyoplast(s) were then aspirated back into the biopsy pipette together with a small amount of HVJ-E in a volume equal to the size of karyoplast. The biopsy pipette was then moved to the drop containing the enucleated recipient embryo and holding pipette re-introduced into drop and recipient embryo immobilised. Using the same hole initially made to allow removal of the PN karyoplasts, the biopsy pipette was the lowered into the enucleated recipient embryo and the karyolpast expelled together with a small volume of HVJ-E to assist with fusion. Here it is important to ensure good contact is made between the karyoplast membrane and the membrane of the embryo cytoplasm to encourage fusion (Figure 4.1e). The embryo is then left within the manipulation drop to allow the karyoplast to fuse with the recipient embryo (Figure4.1f) (can take between 10-60 minutes) (method referenced from Craven et al, 2010).

4.3.5 Embryo culture

When fusion had occurred, embryos were transferred to a fresh culture dish containing G1V5 plus media (Vitrolife, Kungsbacka, Sweden) (50µl drops overlaid with 4ml Ovoil). On the third day of culture manipulated embryos were then transferred to G2v5 plus media (Vitrolife, Kungsbacka, Sweden) (50µl drops). Embryos may be cultured to day 7 where at this time all embryos must be discarded (in 10x EBSS solution) or fixed/frozen for further analysis (Fig4.2).

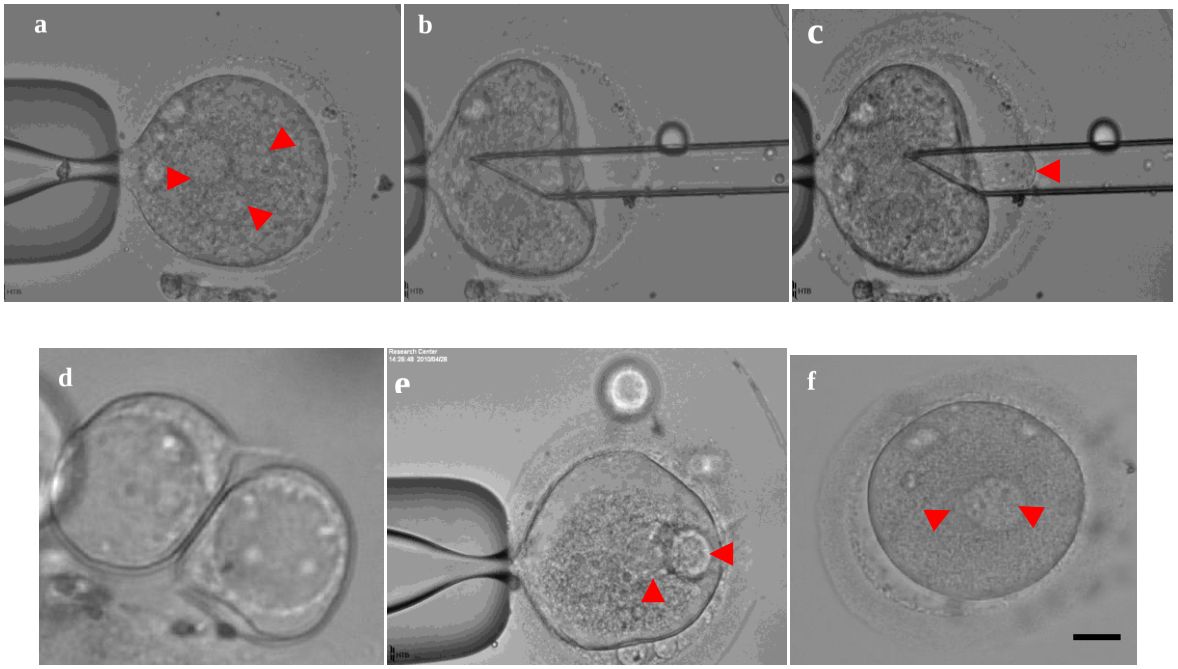


Figure4. 1 Montage of images which represent key stages during pronuclear transfer procedure.

(a) 3PN embryo adhered to holding pipette (b) small hole has been made in the *zona pellucida* and biopsy pipette (c) pronuclear karyoplast at the tip of the biopsy pipette (d) two karyoplasts before transfer to embryo (e) enucleated 3PN embryo, now containing the 2PN karyoplasts (as marked with arrows) from donor embryo (f) Recipient embryo 20-30 minutes following transfer of PN karyoplasts, here both karyoplasts (as marked with arrows) have successfully fused with the cytoplasm of the recipient embryo.

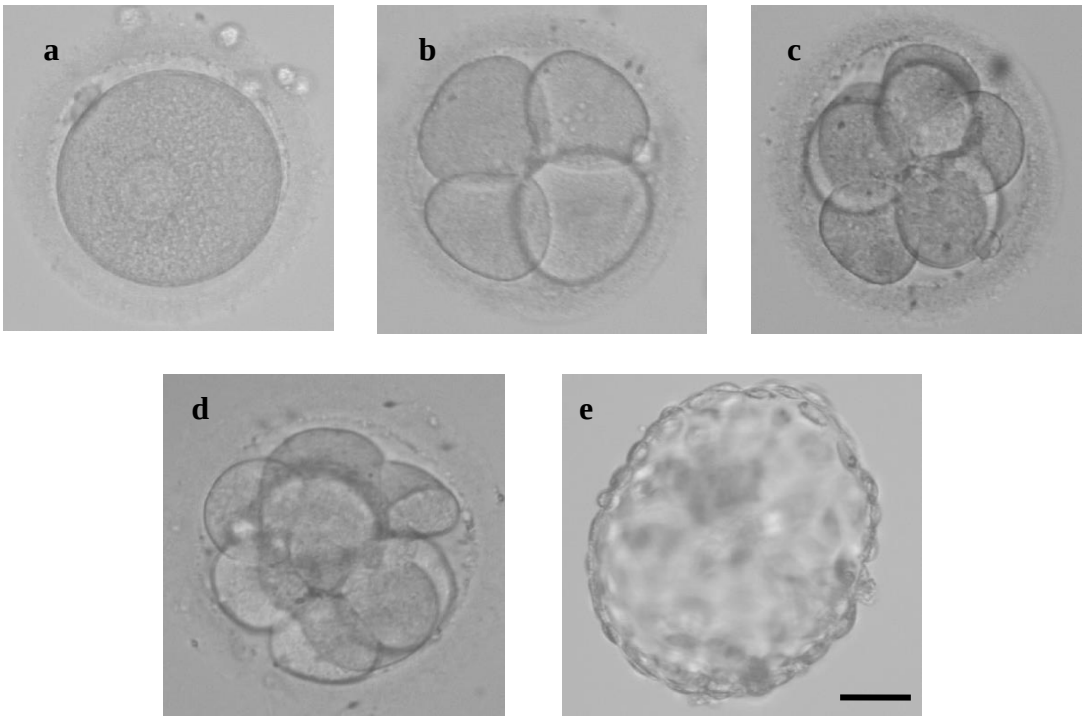


Figure4. 2 Images representing development observed for embryos following pronuclear transfer

Above images represent the different stages of embryo development following manipulation from the initial PN stage embryo though to the blastocyst stage at day 5. Pronuclear stage embryo (a), day 2 4-cell embryo (b), day 3, 8-10 cell embryo (c), 16-24-cell compacting embryo (morale stage) (d), and day 5 early blastocyst (e). Scale bar, 50µm.

4.4 Results

4.4.1 Factors that influence success in pronuclear transfer (PNT)

4.4.1.1 Priming of pipettes

One important aspect of the pronuclear transfer technique is ensuring that both the holding and biopsy pipettes are primed correctly before beginning the manipulation procedure. By priming we refer to ensuring both the holding ad biopsy pipettes have been washed thoroughly so as to remove any old media which or air pockets before then loading the pipettes with fresh media from the manipulating drop. Here care must be taken so as to not overload the biopsy and holding pipettes with media from the manipulation drops as this can result in loss of suction. Conversely a lack of media within the pipette allows air to flow freely and often results in air bubbles flooding the drop the pipettes are currently held within at the time. Ensuring the pipettes are correctly primed is a relatively important aspect of the technique to safeguarding the zygotes during the manipulation procedure.

4.4.1.2 Finding the best plane of view

Another important aspect central to the pronuclear transfer technique is the need to be in the correct plane of focus, before attaching the embryo to the holding pipette and importantly before making a small hole in the *zona pellucida* using the clinical grade laser. As once a hole has been made in the *zona pellucida* you have committed yourself to using that hole as the point of entry for the biopsy pipette, both to limit the number of holes made so as to not damage the integrity of the zygote and limit the number of times you use the laser due to the effect this could pose with regards to onward development potential. Furthermore pronuclei are easier to remove when they are in the correct focus, often resulting in less, disturbance to the cytoplasm hence ensuring the integrity of the embryo and pronuclei are maintained. However it can sometimes be more complicated to ensure that all pronuclei within the zygote lie within the same focus when working with the abnormally fertilised human zygotes (Fig4.3c). The best one can often hope to achieve in this situation is focusing the zygote so that the majority of

pronuclei are within focus, which can often be judged by the sharp contrast of the ooplasmic membrane (Fig4.3c). The focus can then be adjust slightly using the fine focus control during the manipulation procedure so as to assist in the removal of the remaining pronuclei if not possible on the current focus.

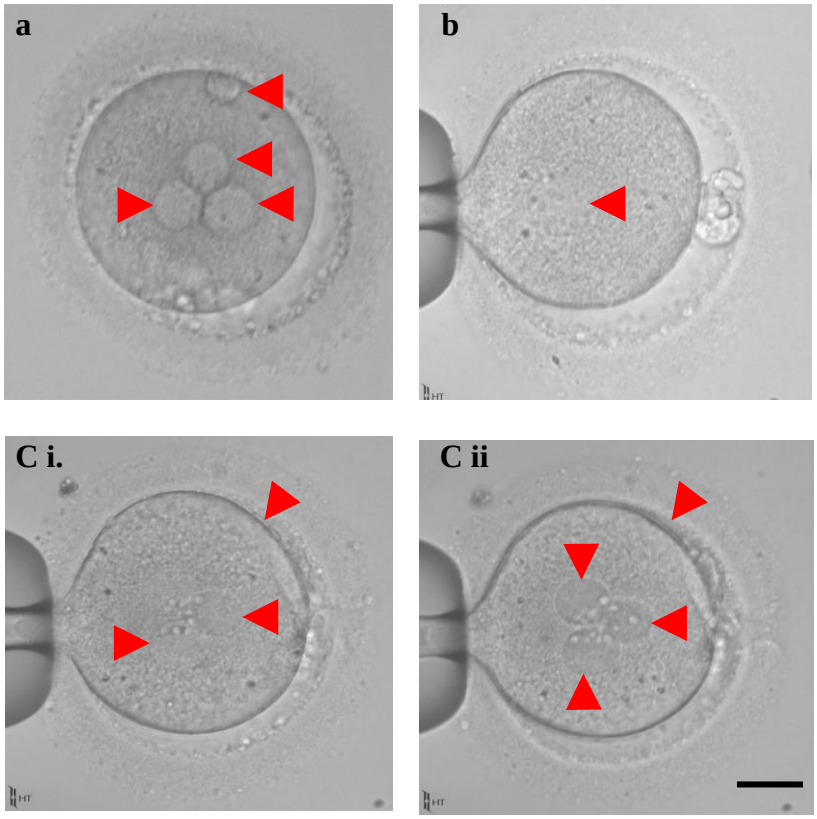


Figure4. 3 Finding the best plane of focus

Prior to beginning the manipulation procedure it is essential that the pronuclei lie within the best plane of focus before attaching the zygote to the holding pipette. However as the images above demonstrate this is not always easy to achieve. (a) tri-pronucleate (3PN) abnormally fertilized zygote in which all 3 pronuclei are visible (as indicated by arrows) the sharp outline of the cytoplasmic membrane and zona pellucida (as indicated by the arrow) would also indicate that this zygote is orientated in the best possible plane of focus to attempt PNT (b) a mono-pronucleate zygote in which the pronucleus is only just visible, indeed faint pronuclei can also make pronucleus removal more difficult (ci) a tri-pronucleate abnormally fertilised zygote in which only two of the three pronuclei can be clearly visualized , however the sharp edge of the ooplasmic membrane (as indicated by arrow) would suggest this zygote is orientated in the best plane of focus (cii) the same a tri-pronucleate zygote (as displayed in image c), in which all 3 pronuclei can be clearly seen however to achieve this the zygote has been orientated so that it is slightly out of focus as demonstrated by the blurry nature outline of both the cytoplasmic membrane ad inner wall of the *zona pellucida* (indicated by arrow). Scale bar, 50µm.

4.4.1.3 Accounting for the presence of polar bodies

Another factor which must also be considered when orientating the zygote is the position and location of the first and second polar bodies within the zygote. The position of the polar bodies must be considered in order to ensure that they do not interfere with the manipulations or pose a risk of fusing back with the zygote following introduction of HVJ-E. Therefore the zygote is usually orientated so that the polar bodies are located at either the 6 or 12 o'clock position (Fig4.3a and Fig4.4a). However by ensuring the polar bodies are located a safe distance from the site of the hole made to allow the PNT biopsy pipette to enter the zygote, the location and focus of the pronuclei can often be compromised. It is also often the case that the polar bodies are located in areas of increased perivitelline space (Figure4.4b) (the area between the ooplasmic membrane and inner wall of the *zona pellucida*) which would prove ideal areas to place the biopsy hole, as the hole is made using a laser diode. The laser is equipped with isothermic rings (Fig4.5) which can be used to ensure that the laser is being used at safe enough distance so as not to inflict damage or detrimentally affect the zygote. However it is often preferential to place the hole in an area of increased perivitelline space, therefore a compromise must be made regarding the location of the polar bodies and the chosen area to make the biopsy hole in the *zona pellucida.*

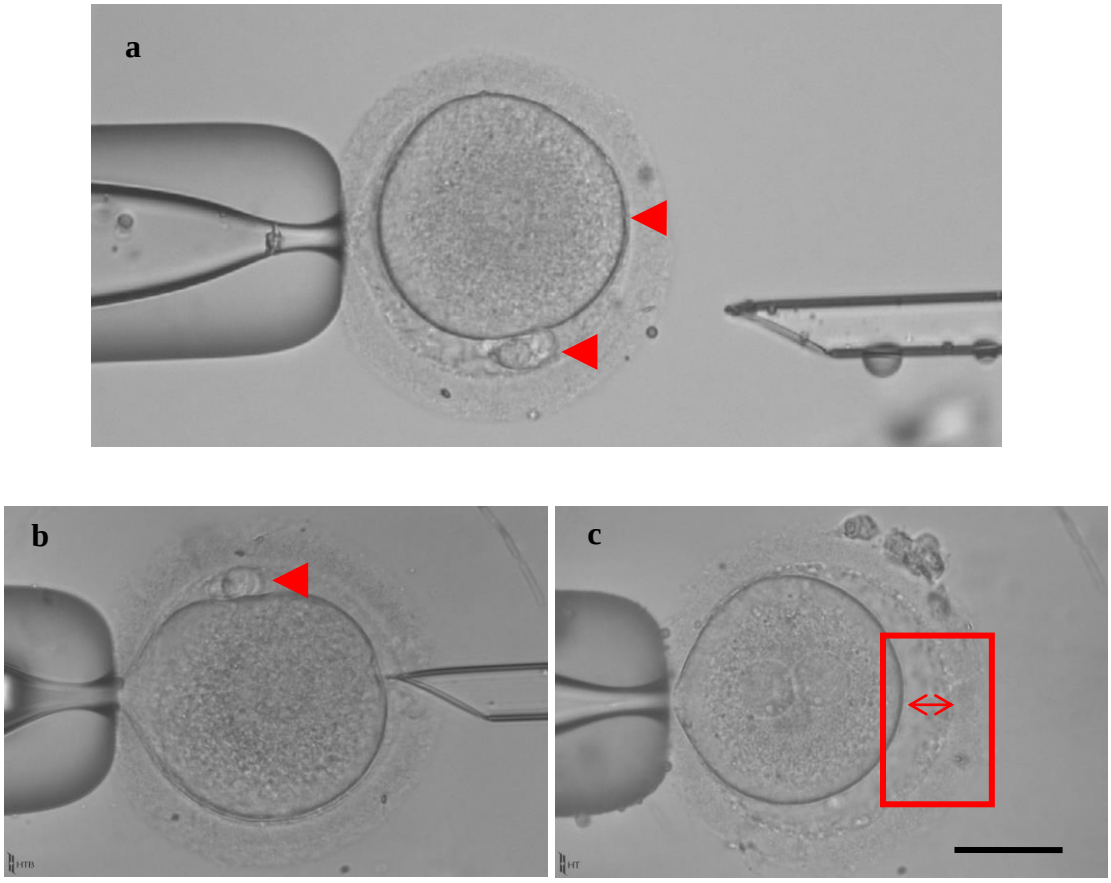


Figure4. 4 Orientating, the zygote with respect to the first and second polar bodies

The position of the polar bodies must be considered in order to ensure that they do not interfere with the manipulations or pose a risk of fusing back with the zygote following introduction of HVJ-E. Therefore the zygote is usually orientated so that the polar bodies are located at either the (a) 6 or 12 o'clock position (indicated by arrow, above). However it is often the case that the polar bodies are located in areas of increased perivitelline space (a and b) also ideal areas to place holes using the laser diode so as to allow the biopsy pipette to gain entry. This ensures that the zygote is not at risk of the laser misfiring and hitting the zygote and also from heat dissipation emitted when the laser is fired. Therefore by placing the holes in areas where there is increased distance between the *zona pellucida* and the outer membrane of the cytoplasm (c) (as indicated by boxed area), it is less likely that the integrity of the zygote could be harmed. However occasionally polar bodies degenerate or are not present, therefore the zygote can be orientated more easily (c). Scale bars, 50µm.

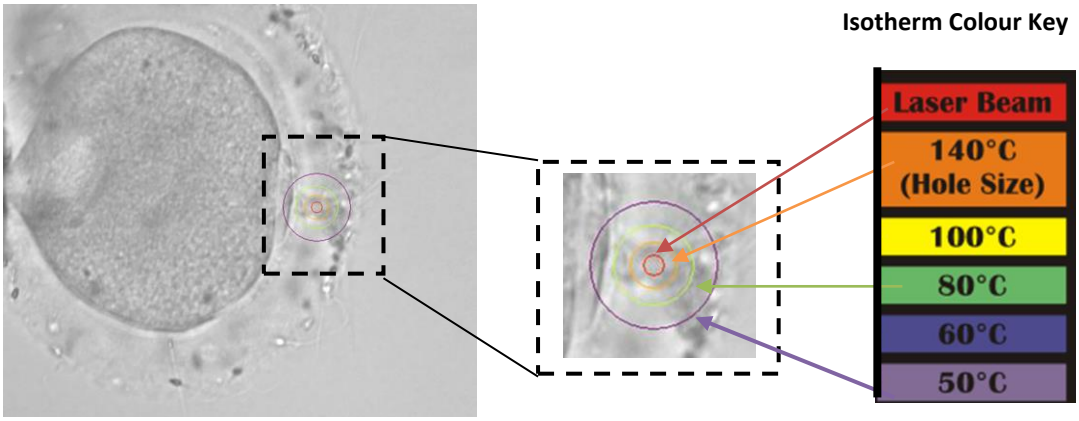


Figure4. 5 The importance of Isotherm rings

Isotherm rings help prevent potential harmful effects posed to the zygote adjacent to the zona due to heat dissipation resulting from laser drilling. The isotherm rings appear on the screen as a series of six concentric circles of varying colours (isothermic colour key, above) and indicate the maximum temperature reached at the ring diameter at various laser pulse durations (right). At longer pulse duration, it is apparent that temperatures radiate farther into the centre of the embryo, increasing the likelihood of damage to the zygote. The orange ring (second from centre) is also a useful indicator of the drill hole, size at the selected pulse duration. (http://www.hamiltonthorne.com/products/lasers/zilostk/safety.htm)

4.4.1.4 Problems encountered during karyoplast removal, transport and fusion

Another issue concerned control over the fine balance of both the biopsy and holding pipettes when removing the pronuclei from the zygote. Once the zygote was adhered to the holding pipette and the biopsy pipette in place to remove the pronuclear karyoplasts it was essential that only fire adjustments were made in the air pressure controlling movement of media within both the holding and biopsy pipette. Sudden increases in air supply to the biopsy pipette can lead to the pronuclei rushing into the biopsy pipette accompanied by a large amount of cytoplasm. However after removal from the zygote it is possible to further manipulate the PN karyoplasts to remove additional cytoplasm and in so reduce the size of the karyoplast before fusing with the recipient zygote. This is usually achieved using the biopsy pipette and applying gentle suction with the air syringe controlling the biopsy pipette whilst the karyoplast is pressed against the side of the zygote and the excess cytoplasm nipped off as a cytoplast and discarded to the edge of the manipulation drop.

The next step is to pick the PN karyoplasts up using the biopsy pipette. However once the karyoplasts have been removed from the zygote they are only surrounded by a small volume of cytoplasm, making them extremely fragile in nature. Great care must therefore be taken when picking up the karyoplasts for this reason, due the potential risk for the karyoplasts to lyse at this time. Several steps can be taken to help minimise the risk of the karyoplasts lysing including reducing the amount of media in the biopsy pipette to prevent the pipette from becoming overloaded with media, this in turn can help increase the overall control over the biopsy pipette. Small incremental increases in air flow can also help prevent karyoplasts from lysing (Fig4.6a).

Once the karyoplasts have been picked up by the biopsy pipette they are then carried to the drop of HVJ-E within the manipulation dish and brought to the end of the biopsy pipette, taking care to not entirely release them into the drop of HVJ-E. Once at the end of the biopsy pipette the karyoplasts are rolled so as to ensure they are coated with sufficient HVJ-E before they are then brought back fully into the biopsy pipette with the addition of a small amount of HVJ-E equal to the size of the PN karyoplast.

The final step, involves transferring the karyoplast accompanied by a small volume of HVJ-E to the recipient embryo. By this time the karyoplasts have been out of the zygote for a considerable amount of time and are now more susceptible to the potential risk of lysing. Therefore great care must be taken when placing the karyoplast into the recipient zygote. To minimise the risk of the karyoplast lysing at this point, the amount of media within the biopsy pipette should be kept to a minimum (the biopsy pipette should contain enough media so that the meniscus is also visible within the pipette), this in turn helps to increase control over the contents of the biopsy pipette. By reducing the volume of media within the biopsy pipette the karyoplast should move towards the mouth of the biopsy pipette (Fig4.6b and c), eventually leaving only a small volume of HVJ-E in front of the karyoplast. The biopsy pipette at this point should be taken towards the biopsy hole of the recipient zygote and gently expelled into the zygote ensuring good contact is made between the karyoplast and cytoplasm of the recipient zygote (Fig4.6d).

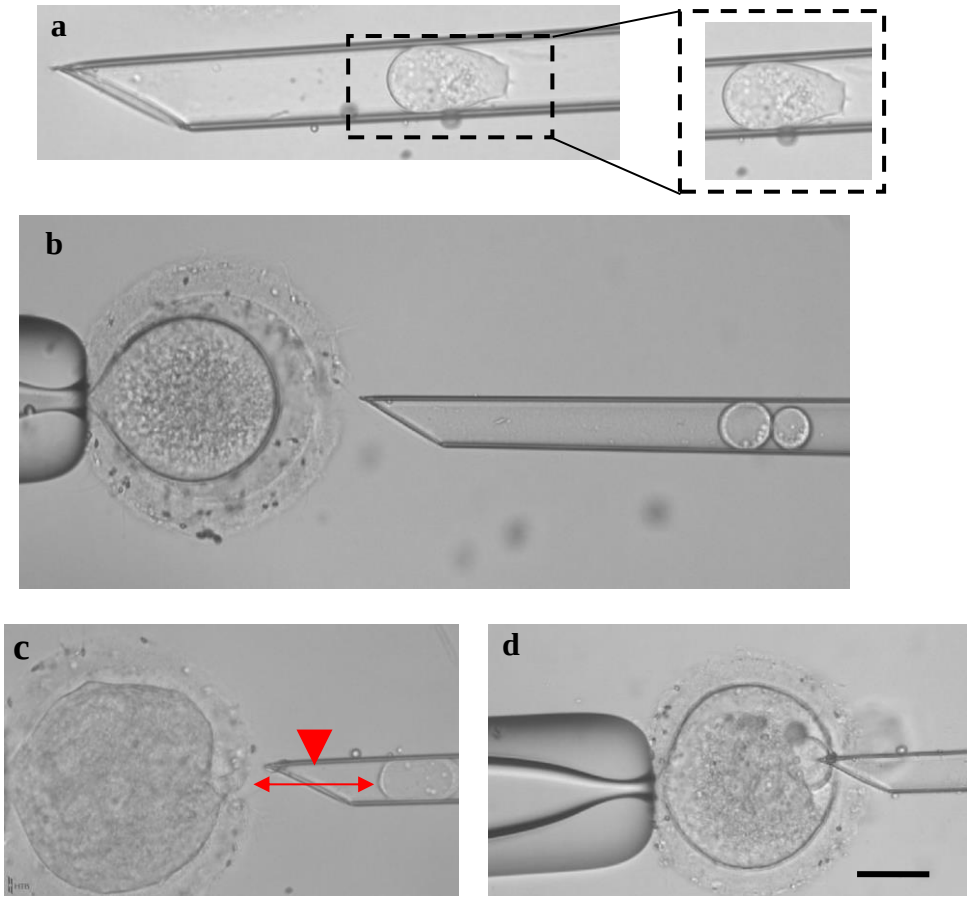


Figure4. 6 Karyoplast removal, transport and fusion

Above images represent different stages during karyoplast removal, transport and fusion. Once the karyoplast has been removed from the donor zygote, it consists of the pronuclei surrounded by a small volume of cytoplasm (so as to minimise the amount of mtDNA which could potentially be carried over to the recipient zygote). Therefore the pronuclear karyoplast has relatively little protection and is highly fragile during the transfer procedure. (a) Great care must be taken when picking up the karyoplast, as sudden increases in air pressure to the biopsy pipette can result in the karyoplast rushing into the pipette and lysing as a consequence. (b and c) Furthermore when preparing to introduce the karyoplast into the recipient zygote it is best to ensure the karyoplasts are relatively close together In the biopsy pipette (b) and ensure that only a small volume of media is presents within the pipette, including to increases control over karyoplast movement (including a small volume of HVJ-E in front of the karyoplasts if possible equal to the size of the pronuclear karyoplasts).The karyoplasts at this point can then be slowly released into the recipient zygote ensuring good contact is made with the cytoplasm to allow for efficient and successful fusion to occur (d). Scale bars, 50µm.

4.4.2 Embryo Development

The aim of this chapter was develop the appropriate skills required to perform Pronuclear Transfer (PNT). Overall I attempted to manipulate a considerable number of abnormally fertilized human zygotes (n=89) during a 6 month period. Here zygotes either received one (n=38) or two pronuclei (n=51) from an enucleated corresponding embryo and development was monitored for all embryos which survived following manipulation. Initially a considerable number of embryos degenerated (n=33) either during or following manipulation procedures due to: removal of karyoplasts before cytoskeletal inhibitors have taken affect, lysing of PN karyoplasts during removal/fusion back with zygotes, loss of control over air syringe controlling the biopsy pipette, resulting in degeneration of the cytoplasm.

Development *in vitro* was used to assess improvement and ability to perform PNT following the few first months from initially being introduced to the technique (April-July). Initially the majority of embryos surviving the PNT manipulation procedure attained development to the <4cell stage 26.3% (April) before finally arresting development (Figure 4.7 and Table 4.1). However with further practice and improved manipulation skills, after performing an increased number of transfers the overall number of zygotes which survived the manipulation procedure did improve. This was marked by the reduction in the number of PNT manipulated embryos attaining development to the <4cell stage 23.5 % (May) and 15.4% (June). In turn the number of embryos attaining development to the 4-8 cell stage and >8 cell stage increased, with the number of embryos developing to >8-cell stage peaking at 70.6% (May). Indeed over the four month learning period data I gathered developmental data for PNT manipulated embryos, I was able to demonstrate a positive trend in regards to development stage attained with a significant number of embryos attaining development to >8-cell stage in comparison to the number who arrested development at the <4-cell stage (Figure 4.7 and Table 4.1).

However there are several factors independent of those associated with operator errors when learning the PNT technique which could also have impacted upon embryo development. This includes issues associated with the equipment used to perform the PNT technique and the fact the embryos utilised for this study were abnormally fertilized human zygotes, which are known to demonstrate a reduced potential for

development which I will discuss in greater detail later in this chapter. However factors associated with the equipment include, the impact of the laser used to make a hole in the *zona pellucida*, the impact of incubation within solutions containing cytoskeletal inhibitors, the fusing agent HVJ-E, extended exposure to non-natural light. This is only a small list of the many technical associated factors which could have contributed to development potential following PNT manipulation. Unfortunately it is difficult to control for all of these factors during each individual manipulation and therefore it is impossible to know how each of these factors independently affect development potential.

Month	Total Number of Embryos Manipulated per month	<4-cell		4-8cell		>8-cell	
		Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)
April	19	(n=5/19)	26.3	(n=9/19)	47.4	(n=5/19)	26.3
May	17	(n=4/17)	23.5	(n=1/17)	5.9	(n=12/17)	70.6
June	13	(n=2/13)	15.4	(n=4/13)	30.8	(n=7/13)	53.8
July	7	(n=0/7)	0	(n=3/7)	42.8	(n=4/7)	57.2
Total	n=56	(n=11/56)	19.6	(n=17/56)	30.4	(n=28/56)	50

Table4. 1 Initial experience of learning the pronuclear transfer procedures with embryonic development as the main outcome measure

(a)Tabular representation of development data acquired to demonstrate progress during learning the pronuclear transfer technique (PNT) for abnormally fertilised human zygotes which successfully survived the PNT procedure and went onto cleave after.

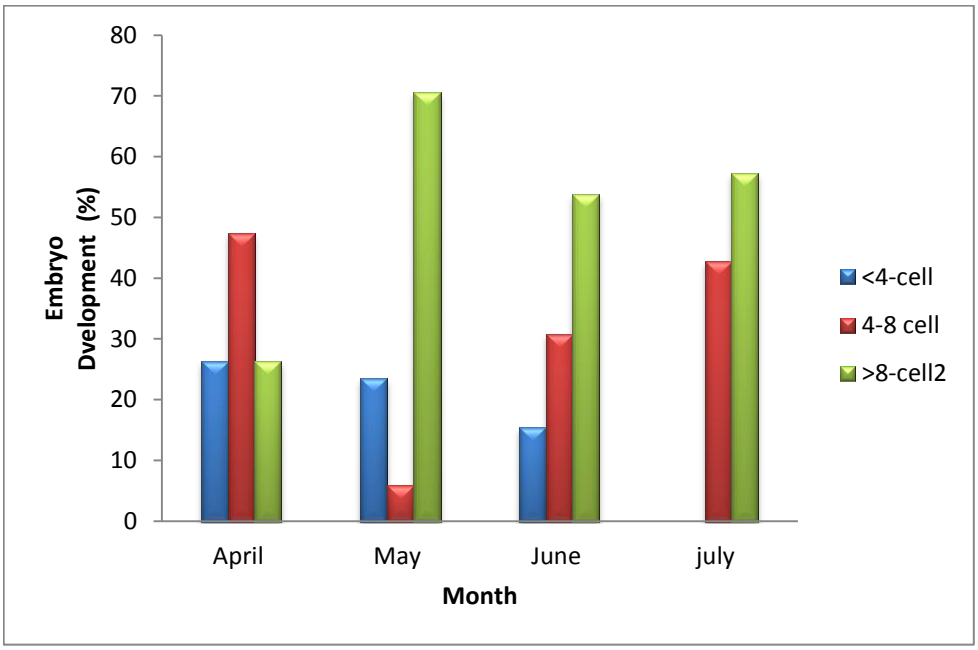
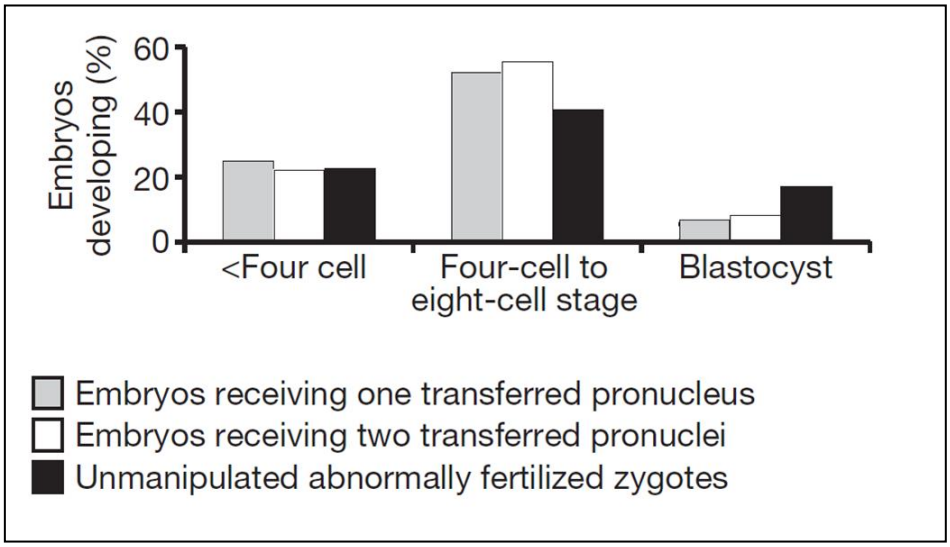


Figure4. 7 Initial experience of learning the pronuclear transfer procedures with embryonic development as the main outcome measure

(a)Above graphical representation of development data acquired to demonstrate progress during learning the pronuclear transfer technique (PNT) for abnormally fertilised human zygotes which successfully survived the PNT procedure and went onto cleave after.

a



b

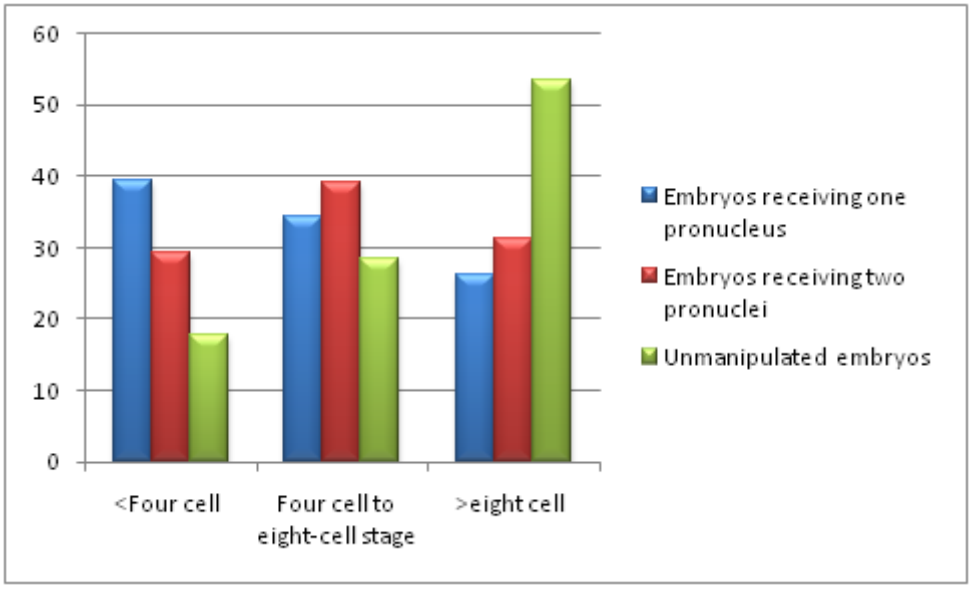


Figure4. 8 Development of unmanipulated abnormally fertilized zygotes following pronuclear transfer

(a) Development data obtained by Dr L Butterworth for pronuclear transfer embryos (b) Above graph represents current development outcomes for both manipulated and unmanipulated embryos. Here we compared development following manipulation where embryos received either 1 (blue) (n=38) or 2 PN (red) (n=51) karyoplasts from a donor zygote. We also compared development in comparison to those embryos which were not manipulated (green) (n=56).

4.4.3 Overall development of pronuclear transfer (PNT) embryos with previously acquired developmental data following PNT

After establishing that I was able to perform the PNT technique, with a significant number of embryos surviving the manipulation procedure intact the next stage involved determining the developmental capacity of pronuclear transfer embryos containing either one or two pronuclei. Here overall development observed was encouraging (Fig4.8) with 10/38 (26.3%) of those embryos receiving one transferred pronucleus and 16/51 (31.3%) embryos receiving two transferred pronuclei going onto develop to at least the eight-cell stage. Furthermore these results are consistent with previous published data for the same technique (Craven *et al*, 2010) (Fig5.8a) published from our lab which showed that following pronuclear transfer around one quarter (n=10/44) of embryos receiving one transferred pronucleus and around the same number/percentage of those (n=8/36) embryos receiving two transferred pronuclei successfully went onto develop to more than the eight-cell stage (Fig4.8). The development data that I attained from these abnormally fertilised zygotes successfully demonstrates the reproducibility of the PNT technique when performed by different operators. This is very important because if the PNT technique becomes available as a clinically recognised procedure it is highly likely that several operators will be required to be trained in the technique. Due to the highly delicate nature of the material involved and the skill required to ensure zygotes survive the procedure given adequate time and experience, it does appear that it is possible to perform the PNT procedure by different operators and importantly achieve highly similar levels of onward development,

Interestingly it was found that a significant number of zygotes which received only one single pronucleus failed to develop beyond the four-cell stage, which was slightly less consistent with previous data. Obviously those embryos only receiving one pronucleus are more likely to have a reduced developmental capacity as they are lacking either the maternal or paternal genome and therefore only contain a haploid set of chromosomes. However previous studies examining abnormally fertilised embryos have shown that some 1PN embryos which contain a rather large single pronucleus can actually be representative of two-fused pronuclei. Here previous studies examined a number of abnormally fertilised embryos and found that a proportion of 1PN abnormally fertilised embryos had a normal diploid karyotype, suggesting the premature fusion of both the maternal and paternal pronuclei in so forming one visible pronuclei

(Feenan and Herbert, 2006) . This could therefore influence the rates we have been observing with regards to compatibility with onward development following manipulation.

4.4.4 Pronuclear transfer (PNT) in normally fertilised zygotes

Experiments to determine the efficiency and feasibility of pronuclear transfer between normally fertilised human zygotes, created from oocytes donated by altruistic donors and fertilised using donor sperm are now underway. This was made possible following the passing of legislation through parliament to the 1990 HFEA ACT so as to allow the creation of normally fertilised human zygotes for the purpose of research. This is essential as all previous experiments to demonstrate proof of principle of the PNT technique have been performed in abnormally fertilized human zygotes. However as development is known to be impaired in abnormally fertilized human zygotes, it is essential that experiments be performed using normally fertilised human zygotes, which contain only two pronuclei and therefore have the correct chromosomal constitution and a greater developmental potential in comparison to their abnormally fertilised counterparts.

Unfortunately one of the altruistic egg donors only managed to produce 1 oocyte and therefore it was not possible to perform reciprocal transfers between the oocytes. However the second altruistic egg donor did produce a significant number of oocytes (n=13), a significant number of which were able to fertilised (n=9), using normal IVF, as these were fresh oocytes unlike the vitrified oocytes, which we will cover in a later chapter (chapter 7) which require intracytoplasmic (ICSI) fertilisation due to zona hardening following cryopreservation and storage in liquid nitrogen. However fertilisation achieved was good, with a number of normally fertilised zygotes (n=8/9) produced, judged by the presence of two pronuclei following fertilisation checks the morning after fertilisation was performed (Fig4.9). Four normally fertilised zygotes were used for the PNT experiment the pronuclei were either removed before they were then fused back with the same zygote or removed from one zygote and fused back with another enucleated zygote obtained from the same patient. Although it was not possible to perform reciprocal transfers between zygotes obtained from two different patients, this did allow us however to ascertain if development observed following PNT may be improved as a result of performing the transfers in normally fertilised zygotes,

known to only contain 2 pronuclei as opposed to all transfer proceeding this, which were performed in abnormally fertilised human zygotes.

The majority of zygotes used for PNT that subsequently survived the procedure, went onto to show signs of onward development *in vitro* (n=3/4) (Fig4.10). At least one of the embryos demonstrated signs of compaction (Fig4.10e), however unfortunately none of the embryos developed to blastocyst stage embryos. As these zygotes were created from oocytes donated by one of the altruistic egg donors, and none of the normally fertilised zygotes created were cultured without manipulating as controls it was not possible to determine if development was undermined due to the PNT procedure or if indeed the quality of the oocytes was responsible for the relatively poor development observed here. This is on-going research and outside the scope of this thesis.

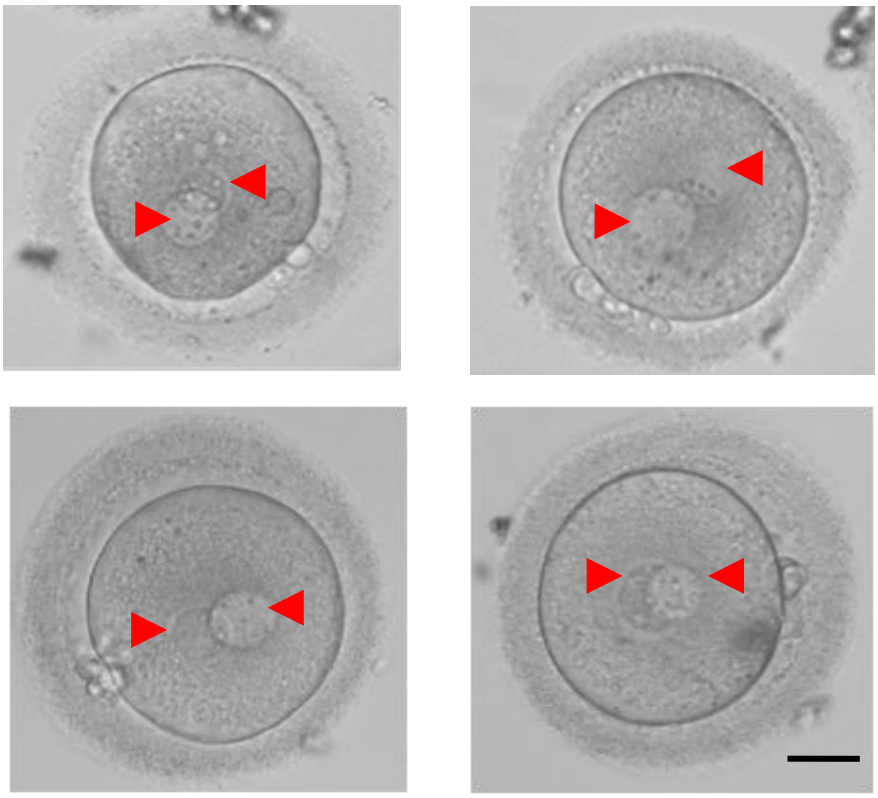


Figure4. 9 Normally fertilised zygotes (containing two pronuclei) following fertilisation checks

Images of normally fertilised human zygotes, as denoted by the appearance of two pronuclei (as indicated by red arrows). These zygotes were created from oocytes donated by an altruistic egg donor which were subsequently fertilised by IVF using donor sperm. Scale bars, 50µm.

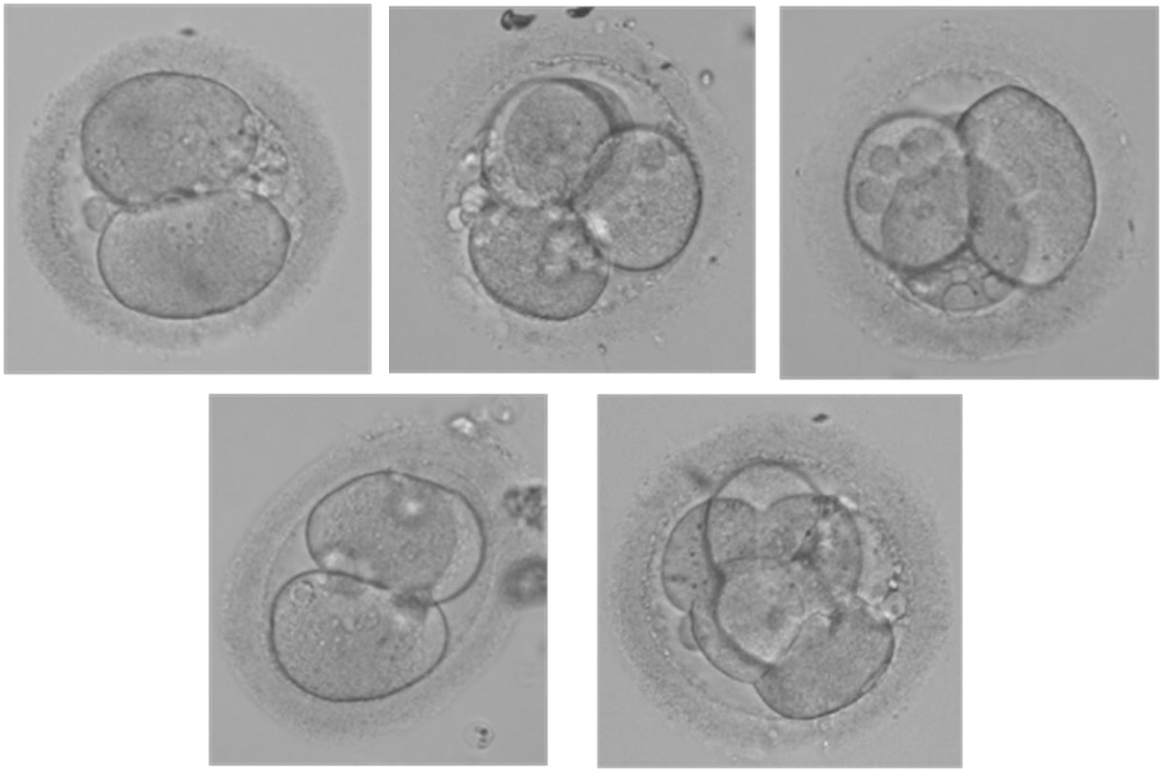


Figure4. 10 Development following PNT between normally fertilized zygotes

Images of embryos which were compatible with onward development following pronuclear transfer (PNT).

4.5 Discussion

At present there is no means available for mothers with homoplasmic or high heteroplasmy mtDNA mothers to conceive healthy offspring which are biologically genetically related. Two nuclear transfer techniques have been proposed to overcome issues associated with risk of transmission from mother to child including; metaphase II spindle transfer (MST) and pronuclear transfer (PNT) (Tachibana et al, 2009; Craven et al, 2010).

Proof of principle studies were performed to examine the feasibility of PNT to prevent the transmission of mtDNA using abnormally fertilised human zygotes. The results of these studies, published in 2010, showed low level of carryover of mtDNA between donor and recipient zygote (Craven et al, 2010). Therefore the aim of this chapter was to examine the reproducibility of the PNT technique between different operators. In order to determine if similar survival and developmental outcomes could be achieved. I was able to develop the appropriate micromanipulation skills required to successfully perform pronuclear transfer (PNT). This technique requires excellent manual dexterity, accuracy and control. Similar micromanipulation is also used to perform, embryo biopsy and intracytoplasmic sperm injection (ICSI).

All manipulations were performed using a Nikon microscope, equipped with the Integra Ti micromanipulation workstation. This uses fine glass pulled pipettes; one holding pipette to secure zygotes and a biopsy pipette to allow removal of pronuclei. The pipettes are controlled by air syringes which allow for suction to be increased/decreased for the correct degree of pressure to be applied to allow pronuclei to be removed effectively and intact. In addition to developing the appropriate skills required to perform the technique, I wished to determine the operator reproducibility.

In developing the appropriate skills required to perform the PNT technique, I identified several key observations and issues associated with performing the PNT technique. This included orientation polar bodies of the zygote, the presence of fragmentation within the perivitelline space, visibility of the pronuclei within the zygote, the orientation of the pronuclei and also the efficiency of the cytoskeletal inhibitors. Together these factors were able to complicate the nature of the procedure and also had the ability to affect outcomes following manipulation. For example the efficiency of the

cytoskeletal inhibitors during the procedure, impacted upon both the ability to remove karyoplasts efficiently and intact and also the ability to manipulate karyoplasts further once outside of the zygote. The production of larger karyoplasts inevitably means they will contain more cytoplasm and so would also be expected to contain more mitochondria from the donor embryo, resulting in the potential for higher levels of mtDNA carry-over.

However a number of the issues I identified to be associated with the technique was due to the fact that abnormally fertilized human zygotes were used as the main sample source for these studies. These embryos are the result of abnormal fertilization and therefore do not contain the correct chromosome complement. The majority of the zygotes I used in this study contained either 1 or 3 pronuclei. Some multipronuclear zygotes contained >4 pronuclei and these proved incredibly difficult to remove successfully. This is because the pronuclei were often orientated in different planes of the zygote. Furthermore the time at which the abnormally fertilized human zygotes were obtained from the clinical IVF laboratory after fertilization checks meant that sometimes the pronuclei in some zygotes were already faint. Therefore by the time I attempted to use the zygotes to manipulate not all of the pronuclei were still visible. This was a particular problem associated with the multipronuclear zygotes and on occasions meant that it was not possible to remove all pronuclei.

It took several months to acquire the appropriate skills required to successfully perform the PNT technique. Overall survival and developmental potential during this time was low with a considerable number of zygotes degenerating during and following the procedure. However both survival and potential for development began to increase with further experience with the technique.

However analysis showed that a number of zygotes demonstrated potential for onward development to the 4-8 cell-stage (n=33). However, development to the blastocyst stage *in vitro* was low at ~3% compared to the previous blastocyst development rate achieved of 6.8% for single pronuclear and 8.3% of double pronuclear transfers (Craven et al, 2010). This could be a result of differences in technique between operators as mentioned previously however all manipulation were performed using abnormally fertilised human zygotes, which would never be replaced due to doubts over

chromosome complement. It is also known that abnormally fertilised zygotes demonstrate a limited potential for development (Fennan and Herbert, 2005).

However issues associated with using abnormally fertilized human zygotes for the purpose of the PNT technique could be overcome if it was possible to use normally fertilised human zygotes instead. Performing transfers in normally fertilized zygotes would allow for more reliable examination of the effect of the PNT technique and the effect it alone has on embryo development. Indeed experiments are now underway to examine the potential of PNT in normally fertilised human zygotes. A number of altruistic donors have been recruited to donate oocytes, which will then be fertilised using donor sperm and in so, if all goes well lead to the creation of normally fertilised zygotes, containing two pronuclei. Following the creation of these zygotes they are then utilised for PNT experiments and there compatibility and potential for onward development. As this embryos will only contain one maternal and one paternal pronuclei, theoretically they should contain the correct chromosomal constitution and therefore development potential able to be attained should reflect this.

The impact and general use of cytoskeletal inhibitors is also something which may be worth investigating further. Currently two cytoskeletal inhibitors are used during the PNT prcocedure, each of which inhibits different parts of the cytoskeleton. This includes *Cytochalasin B* (CB) and *nocodazole* (noc). CB has been shown to bind to actin filaments and block polymerization and elongation of the actin disrupting microfilament structure. CB has also able to induce polyploidy and also prevent polar body extrusion (Snow, 1973; Siracusa et al., 1980; Bos-Mikich et al., 1997). Nocodazole is an inhibitor of tubulin polymerization and causes breaks in the metaphase spindle and, thus, is often used for cell cycle synchronization experiments (Johnson et al., 1988). There is evidence that treatment with these cytoskeletal inhibitors, provided at the correct concentration and exposure time, development to the blastocyst stage is unaffected *in vitro* (Siracusa et al., 1980; Epstein, 1986; McGrath and Solter, 1984b; Surani et al., 1984). However given the cytotoxicity of the current inhibitors used (cytochalasin B and nocodazole) for the PNT procedure it may indeed be important to investigate the potential of other cytoskeletal inhibitors. It is also worth noting that neither; CB or Noc are available in clinical grade forms this would therefore prevent their use if PNT were to be translated into clinical treatment.

Indeed there are several features which can influence the choice of cytoskeletal inhibitor, including; low toxicity, fast acting and reversibility. These characteristics should be considered when exploring other potential inhibitors. Other compounds which could include be investigated includes other member of the Cytochalasin family (A, B, C, D, E, F, H and J) or even different compounds altogether including colchicine, colcemid and Latrunculin. Several studies have now examined the function and effect of two of the members of the latrunulin family, including; Latrunulin A (Lat A) and Latrunculin B (Lat B) (Walter et al, 2000). Both of these compounds have been shown to demonstrate similar modes of action, inducing actin depolymerization both in vitro and in vivo (Spector et al, 1983; Morton et al, 2000). There is also evidence of Lat A treatment of embryos (Himaki et al, 2010). They studied embryos treated with either Lat A or CB and found that cleavage rate following exposure to Lat A was significantly higher than that achieved for embryos exposed to CB (Hikami et al, 2010). It would therefore prove interesting to examine the use of Latrunculin as an alternative cytoskeletal inhibitor in place of cytochalasin and nocodazole. Although it would be important to assess its effect on subsequent embryo development and mtDNA carryover, in order to determine its efficiency compared to currently used cytoskeletal inhibitors.

However the main aim of this part of work was to demonstrate that the PNT technique can be reproduced between different operators and furthermore similar levels of development observed for these manipulated zygotes. This was important to demonstrate as if the PNT procedure is ever allowed to be used a clinical IVF procedure, it will be important to train others in the procedure so that it can be reproduced. Other groups have examined other variants of the nuclear transfer technique for the purpose of preventing the transmission of mtDNA disease. This of course refers to meiotic spindle transfer (MST). This has now been reported to have been performed in both primates from which live offspring resulted and also more recently human oocytes. Reproducibility has been demonstrated for the MST nuclear transfer technique, demonstrating that both techniques are able to be performed by different operators.

4.6 Summary

- Here I was able to develop the appropriate micromanipulation skills required to successfully perform pronuclear transfer (PNT). This was a technique which I had no previous experience in and required excellent manual dexterity, accuracy and control. However with further practice and gaining experience in the technique, the number of zygotes which survived the manipulation procedure gradually improved (n=89). After demonstrating I was able to successfully perform PNT as judged by the number of zygotes which survived following manipulation the next stage involved determining the developmental capacity of pronuclear transfer embryos containing either one or two pronuclei. For those embryos which successfully survived the pronuclear transfer procedure a significant number showed onward development.

- As part of developing the appropriate skills to perform the PNT procedure I identified a number of key factors involved in attaining successful PNT manipulations. Hopefully by identifying a series of key factors it may facilitate in the training of future individuals in the PNT technique.

- I was also able to perform pronuclear transfer experiments between normally fertilized human zygotes, which showed potential for onward development. These are experiments which are now being explored in greater depth and on a larger scale which are out of the scope of this current thesis.

- There are several aspects of the PNT technique which still require optimisation. This includes the fate of the polar bodies following PNT. Here inactivated viral envelope protein of the Hemagglutinating virus of Japan (HVJ-E) is used to mediate fusion between the pronuclear karyoplast and the recipient zygote. However it has been suggested that if the HVJ-E was able to persist within the perivitelline space following karyoplast fusion that this may allow for the polar bodies to fuse back with the zygote. Therefore I was involved in conducting a pilot study to determine if polar body removal could prevent this additional risk. The results of which may have consequences for how the PNT technique is performed in the future.

Chapter 5 Modifications to the Pronuclear Transfer Technique

Chapter 5 Modifications to Pronuclear Transfer Technique - Polar body biopsy

5.1 Introduction

Polar bodies are sometimes referred to as the 'genetic dustbin' of the oocyte. They are the structures formed following the first and second meiotic divisions, into which the extra set of chromosomes, are extruded, allowing the oocyte to maintain a haploid state in readiness to be fertilised (Magli et al, 2004).

Polar bodies are unique in that they provide a reciprocal image of the chromosomal make-up of the MII oocyte, which is increasingly being used as a diagnostic screening tool to assess not only the ploidy of an oocyte but also test for single gene disorders prior to fertilisation (Verlinsky et al, 1990). This gives couples with a high reproductive risk a better chance of delivering a healthy infant (Magli et al, 2004). Unfortunately neither paternally derived defects nor aneuploidy events, generated at fertilization or after the first cleavage divisions can be diagnosed using the polar body (Kuliev et al., 2003; Magli et al, 2004).

Several studies have evaluated the effect of second polar body removal on the viability and developmental potential *in vitro*, with the majority demonstrating no significant differences in the percentage of morphologically normal blastocysts produced following PB biopsy (Kaplan et al, 1995). This indicates the relatively low impact polar body biopsy exerts upon development and successfully maintaining the integrity of the oocyte. However the exact method used to remove the polar body can influence future outcomes. Despite the independent nature of the polar bodies with respect to the embryo great care must always be taken not to damage the oocyte/embryo during biopsy procedures, as doing so could lead to loss or induced damage to the chromosomal material (Montag et al, 1998). Indeed it has been reported that an estimated 28% of oocytes which undergo polar body biopsy for genetic screening procedures have to be discarded as a result of technical failures during the biopsy or following analytical procedures (Reubinoff and Shushan, 1996).

The type of instrument or pipette used to retrieve polar bodies can also vary between labs. Sharp aspiration needles have routinely been used as the tool of choice to mechanically breach the *zona pellucida* (ZP) and retrieve the polar body simultaneously. However laser-assisted microdissection is now viewed as the preferred method, as this allows for the ZP to be breached in a more controlled manner. Furthermore the use of a laser to breach the ZP enables the use of blunt-ended micropipettes, which reduces risk of potential damage to the oocyte (Montag, 1998). However there was concern raised that the use of a laser diode may result in longer lasting effects which could subsequently impair embryo development. Several studies have now successfully demonstrated that use of a 1.48µm diode laser system is a safe an efficient method to use for laser microdissection of the ZP; which does not impair later embryo development (Rink et al, 1996; Germond et al, 1995; Montag et al, 1998).

Oocytes and embryos in which the *zona pellucida* has been breached manually has been shown to facilitate in the hatching process (Montag et al, 1998). The hole made using the laser/acid Tyrode's to allow for polar body removal frequently results in embryos hatching earlier than would normally be expected if the process was to occur naturally as a result of the *zona pellucida* thinning to allow hatching of the blastocyst. However this does not appear to affect the embryo developmentally or otherwise other than to assist it to hatch occasionally in advance of those embryos which do not have a hole in their *zona pellucida*. Indeed laser assisted hatching is a technique employed within many embryology clinics to aid in blastocyst hatching, primarily in those cases where the *zona pellucida* does not appear to be thinning as would be expected (with respect to expected development) and therefore intervention is required. This is often the case in frozen/vitrified embryos, where the ZP hardens as an artefact of the cryopreservation process.

5.1.1 Explanation for removing the polar bodies

Current work is examining the potential of pronuclear transfer (PNT) as a potential means by which to prevent transmission of mtDNA disease to future offspring in those women whom carry mtDNA mutations at high levels. Proof of principle experiments, already conducted within the lab using abnormally fertilised human zygotes demonstrated that these PNT embryos were compatible with onward development, even

when complicated by the fact that abnormally fertilised zygotes have limited potential for development to the blastocyst stage (Craven et al, 2010). The PNT technique involves the removal of pronuclei from a donor zygote within a karyoplast containing a small volume of cytoplasm. Karyoplasts are then placed under the ZP of a recipient (previously enucleated) zygote and fused using inactivated viral envelope proteins of the Hemagglutinating virus of Japan (HVJ-E).

HVJ-E is a purified product prepared through complete inactivation of Sendai virus (HVJ Hemagglutinating Virus of Japan) through which only the cell membrane-fusing capability of the envelope protein of Sendai virus is retained. The genomic RNA of the Sendai virus contained in HVJ-E has been inactivated completely and therefore has neither infective nor proliferative potentials in cells (as demonstrated schematically in Fig5.1). Studies have previously reported the removal of polar bodies when performing nuclear transfer experiments (Li Meng et al, 1997; Dinnyes et al, 2000; Simerly et al, 2004). As it was suspected that following the addition of HVJ-E to assist in cell fusion, polar bodies with fully intact membranes could also potentially fuse back if sufficient HVJ-E was present. This was determined by the presence of two pronuclei following fertilisation checks. However in order to ensure sufficient activation is achieved electrofusion is often also employed, this it has been said would be enough to allow polar bodies to fuse back with the oocyte. I therefore performed a pilot study to determine if polar body removal could prevent this additional risk incurred by the procedure.

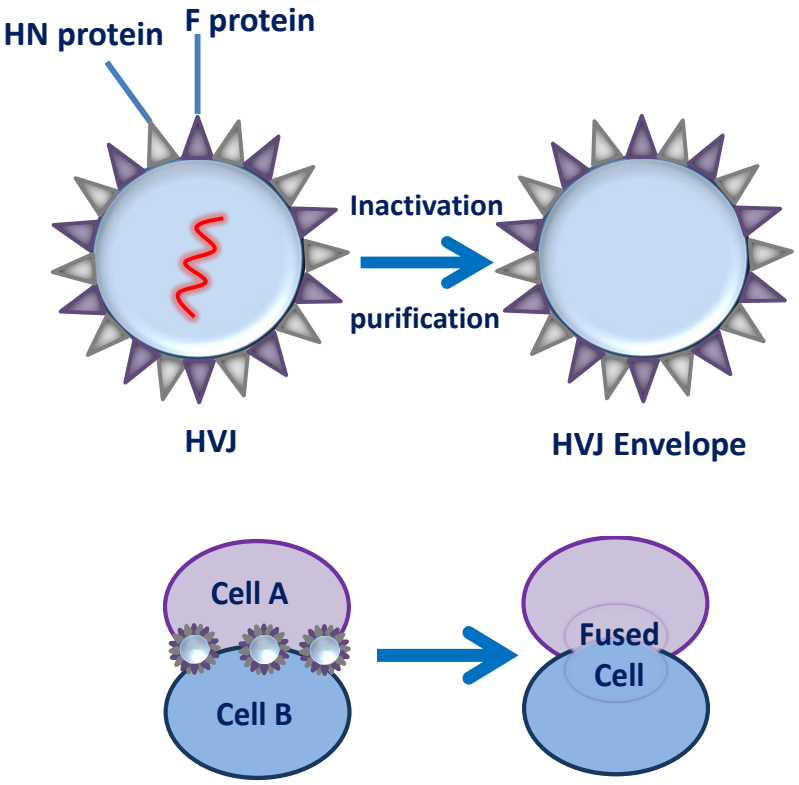


Figure5. 1 Hemagglutinating Virus of Japan (HVJ-E)

Above schematic diagram demonstrates how the genomic RNA of the Sendai virus contained in HVJ-E has been inactivated completely and has neither infective nor proliferative potentials in humans. The HN protein of the HVJ envelope binds specifically to the sialic acid receptor on the cell membrane of the target cell. Membrane fusion is induced by the F protein contained in the HVJ envelope. Allowing fusion to occur between the two, membrane enclosed cells (Adapted from original image www.cosmobio.co.jp).

5.2 Materials and Methods

5.2.1 Removal of polar bodies

Zygotes were transferred to a manipulation dish containing 4µl drops of G1v5plus (Vitrolife, Sweden) overlaid with 3-4ml of Ovoil (Vitrolife, Sweden). The holding and zona drilling pipette were lowered into the manipulation dish and both pipettes primed before use in a spare side drop within the dish. A small hole was made in the *zona pellucida* (ZP), enough to allow the zona drilling pipette to enter the perivitelline space. Once the polar bodies were in the best plane of focus, we could move to the laser objective (x40). The zona drilling pipette was moved towards the first polar body and very slight suction applied using the air syringe. At this point great care must be taken not to apply too much suction as this can result in damage to the embryo. Once the polar body was in the mouth of the zona drilling pipette, we began to ease the pipette out of the zygote, at the same time gently applying suction so as to not lose the polar body or allow it to traverse back towards the zygote (Fig5.2). Once in the pipette the polar body was placed at the outer edge of the drop and the procedure repeated to remove the second polar body.

5.2.2 Embryo culture

When fusion had occurred embryos were transferred to a fresh culture dish containing G1V5 plus media (Vitrolife, Kungsbacka, Sweden) (50µl drops overlaid with 4ml Ovoil). On the third day of culture manipulated embryos were then transferred to G2v5 plus media (Vitrolife, Kungsbacka, Sweden) (50µl drops). Embryos may be cultured to day 7 where at this time all embryos must be discarded (in 10x EBSS solution) or fixed/frozen for further analysis

5.2.3 Immunostaining of blastocysts

Blastocysts were fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature and then transferred to 1% PBS (can be stored once fixed in PBS for 3-4 months). Blastocysts were permeablised for 1 hour at RT using 0.2% Tween and 0.2% Triton X solution in PBS and then blacked in 1% milk in PBS for a 1 hour. Blastocysts

were incubated in the primary goat anti Nanog antibody (Abcam ab21603). (1:1000) overnight in the fridge. The following morning blastocysts were washed three times in PBS, 5 minutes each. Incubated with donkey anti- goat secondary Alexa fluorophores (Invitrogen) diluted 1:1000 in PBS. Sequential washes were then performed (5mins, 10mins and 30mins) in PBS. Finally they were transferred to a 3µl drop (PBS 1:1000 DAPI) overlaid with 1 ml of oil in a glass bottom viewing dish, for viewing.

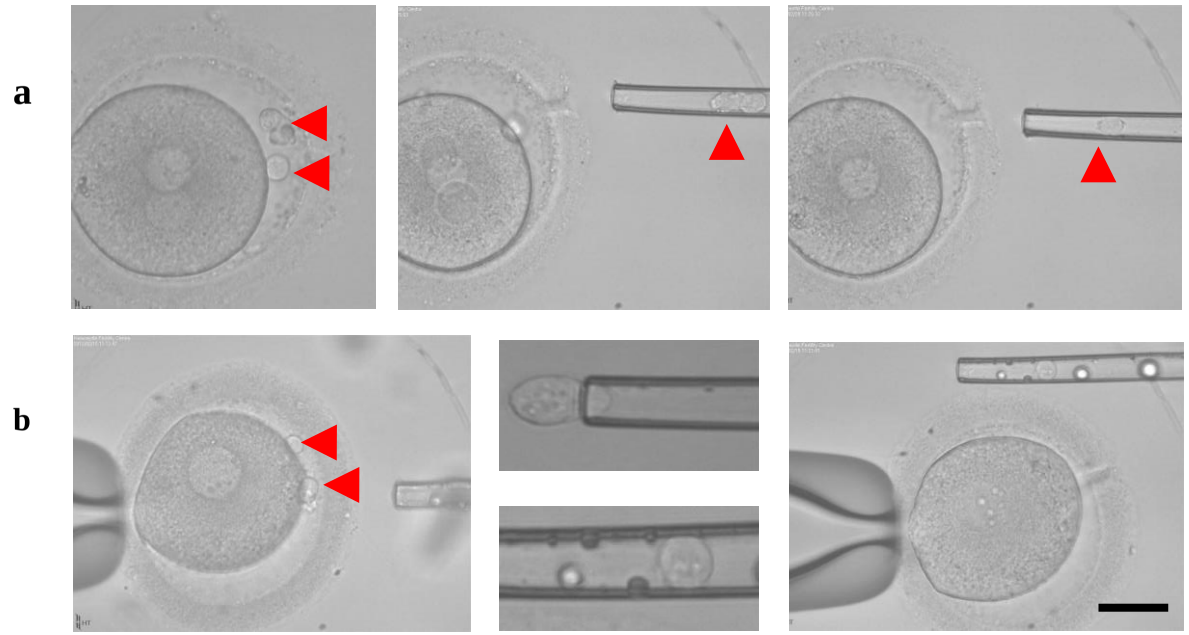


Figure5. 2 Examples of Polar body removal from abnormally fertilised human zygotes

(a) The first image depicts a 3PN embryo from which the polar bodies were biopsied from. A small hole was made in the ZP, for which to allow the biopsy pipette to gain entry into the perivitelline space. For this particular zygote it took two attempts to remove all of the polar bodies, due to their orientation within the perivitelline space. (b) The second two images depict the removal of polar bodies from another (mono-pronucleate) abnormally fertilised zygote. Here the polar bodies were not as fragmented however it was still not possible to remove them at the same time. Scale bar,50µm.

5.3 Results

5.3.1 Problems encountered when learning the PNT technique

5.3.1.1 Difficulties in orientating the zygotes

Our aim was to remove the polar bodies, prior to attempting removal of the pronuclei. However we soon encountered several problems when beginning to examine the potential to remove the primary and secondary polar bodies from the abnormally fertilised human zygotes. Initially we found that as these were abnormally fertilised human zygotes, the morphology of the embryos and their subsequent polar bodies, was occasionally very poor, which complicated the situation. The location of polar bodies within the perivitelline space was irregular, polar bodies were often found on different planes within the zygote and occasionally at different locations (Fig5.3). The often random location of the polar bodies made it incredibly difficult to orientate the zygotes, so that both polar bodies were in a position which they could easily be removed without causing potential damage to the zygote. Furthermore of those abnormally fertilised zygotes we received some had been fertilised using IVF and others using intracytoplasmic sperm injection (ICSI) (Fig5.3). This induced additional problems as those oocytes which had been fertilised using ICSI often contained polar body-like structures at the 6 and 9 o'clock positions of the perivitelline space. This we believed to be an artefact of the ICSI technique since when oocytes are orientated so they are in the correct position to inject with the sperm, they are positioned in so the polar body is out of the way, either at the 12 or 6 o'clock position. The needle used for ICSI is thin and sharp, allowing it to penetrate the *zona pellucida* without the need for a hole. The sperm is therefore injected once a cytoplasmic 'snap-back' is observed, which signifies that the oolema (membrane around the cytoplasm) has sealed back trapping the sperm in the cytoplasm. However on withdrawal of the ICSI pipette from the cytoplasm of the oocyte, a cytoplasmic bridge is sometimes formed which can create 'polar body-like' structures within the perivitelline space (Fig5.3b). It is these structures which we believe were making it difficult to orientate the zygotes, as we could not be sure which, were the actual polar bodies.

As the aim of the study was examine the potential to remove the pronuclei following removal of the polar bodies, whilst minimising the number of holes made in the *zona*

pellucida of the zygote. This influenced the orientation the zygote was positioned in prior to attaching to the holding pipette. The ideal scenario would allow for a single hole to be made in the *zona pellucida* which would allow to, firstly remove the polar bodies and then secondly remove the pronuclei. However t due to the varying positions of the polar bodies in the perivitelline space and the varying planes within the zygote the pronuclei resided this often proved difficult.

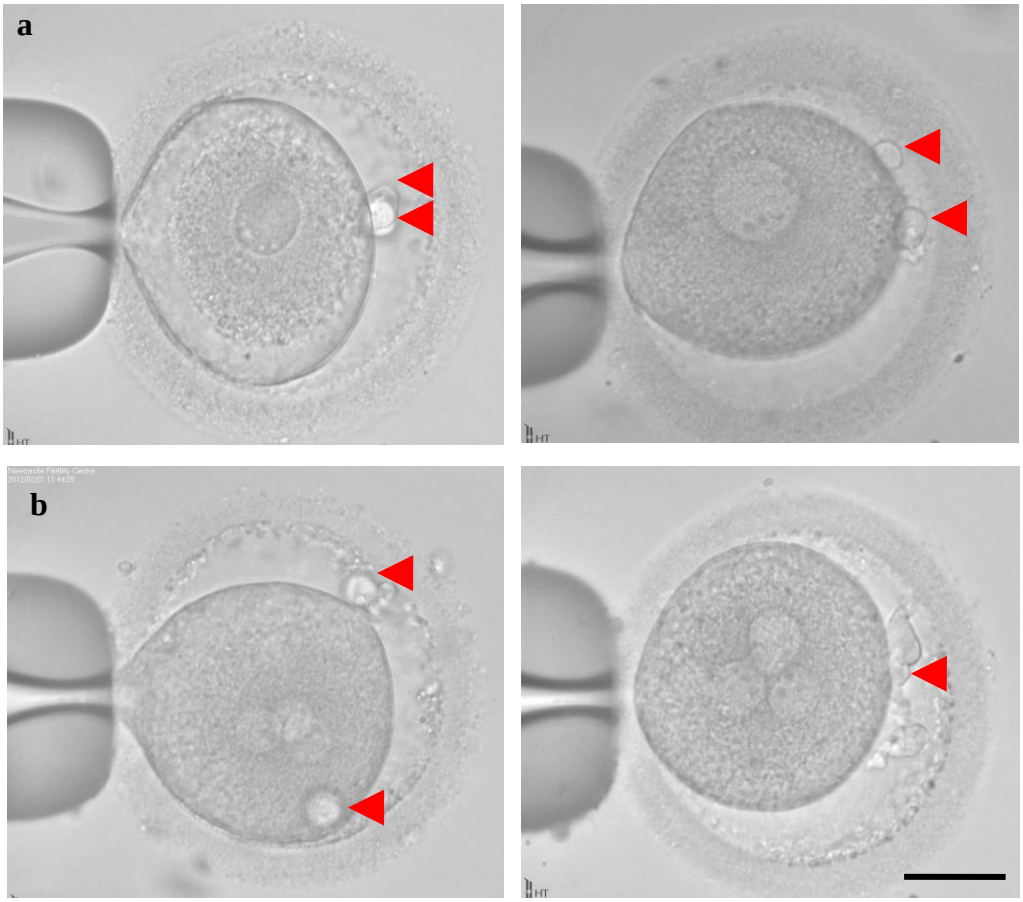


Figure5. 3 Morphological differences observed in polar bodies found in IVF inseminated zygotes versus ICSI fertilised zygotes.

Above images represent observational differences made regarding polar body morphology and position between embryos which were inseminated using straightforward IVF (in vitro fertilization) and those fertilised using ICSI (intra cytoplasmic injection) (a) Polar bodies found in those embryos created following IVF insemination often were found to contain atypical polar bodies, with the first and second polar bodies in approximately the same location (indicated by arrow). (b) However in those embryos created following ICSI, we often found that at least one of the polar bodies was fragmented (indicated by arrow) and often further apart within the perivitelline space (indicated by arrow), making them incredibly more difficult to remove. Scale bar, 50µm.

5.3.1.2 Fragmented polar bodies

A number of zygotes contained fragmented polar bodies, an issue which related to both these being abnormally fertilised zygotes and the time span in which we received them. In particular the primary polar body is formed before the oocyte is fertilised and therefore we would expect this to be more fragmented than the secondary polar body due to the extra time this has been present for (Fig5.4).

Another problem encountered, when removing some of the polar bodies was the ability to tease the polar bodies into the zona drilling pipettes (used for their removal), due to what appeared to be 'cytoplasmic like-bridges' invisible ties existing between the polar body and the zygote. Once the polar body had been successfully navigated the zona drilling pipette it often began to drift back out of the pipette and towards the zygote. To overcome this issue the air flow from the air syringe attached to the biopsy pipette was gradually increased in an attempt to counterbalance the pull back towards the zygote. However on several occasions when the air flow was increased too suddenly the polar body often flew up the zona drilling pipette but also with accompanying cytoplasm from the zygote. Most of those embryos in which the cytoplasm of the zygote was nicked by the pipette went on to degenerate (n=10/39).

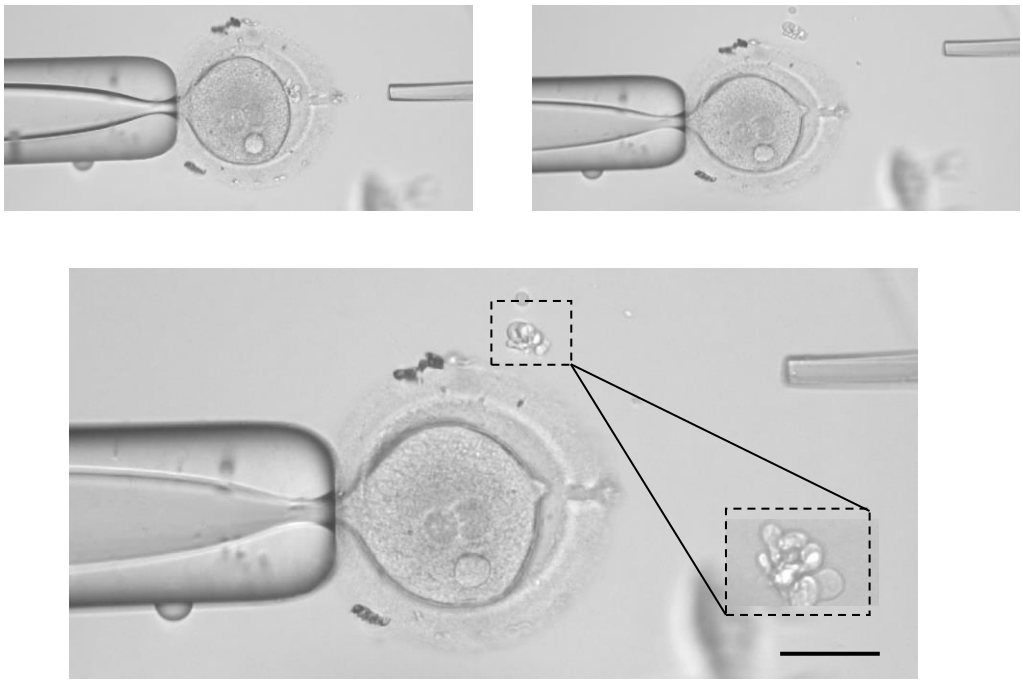


Figure5. 4 Polar body biopsy of fragmented polar body

Above image represents a typical fragmented polar body we attempted to biopsy from an abnormally fertilised human zygote. Scale bar, 50µm.

5.3.2 Polar body biopsy prior to PNT

I attempted to perform polar body biopsy immediately prior to performing PNT on the same zygote. Two different approaches were taken to examine the possibility of performing polar body removal in conjunction with PNT. As part of the first approach polar bodies were removed in media supplemented with cytoskeletal inhibitors (cytochalasin B (1µg/ml) and 1µl nocodazole (2µg/ml) (Sigma-Aldrich, UK)). This allowed for PNT to be performed immediately following polar body removal. For the second approach, zygotes were placed into un-supplemented G1v5plus media for polar bodies removal. Following successful polar body removal these zygotes were then transferred to a different drop of media within the manipulation dish containing media supplemented with the cytoskeletal inhibitors to allow for PNT to be attempted. A comparison was then made between the two different approaches so as to determine, which approach was most feasible to perform ad gave best survival outcomes.

5.3.2.1 Orientation of the zygotes for optimum polar body combined with sequential pronucleus removal

Prior to beginning the procedure (polar body removal followed by PNT) it was often found incredibly difficult to orientate the zygote so that both the polar bodies and the pronuclei were all roughly within the same focus. This was made more difficult when the polar bodies were fragmented and located in different areas within the perivitelline and present in multiples (Fig5.4). It was therefore decided that the primary aim was to ensure that the pronuclei were removed intact and therefore were in the best possible focus. Once the zygote had been orientated in to an acceptable working position, the zygote was attached to the holding pipette, using suction. The zona drilling pipette used for the polar body biopsy procedure was then lowered into the drop of media containing the zygote. A small hole was made in the *zona pellucida* using the laser and the biopsy pipette brought to the mouth of the hole. Using the air syringe controlling the biopsy pipette light suction was applied to encourage the polar body into the mouth of the biopsy pipette. For those zygotes which polar bodies had successfully been removed using the zona drilling pipette, it was necessary to remove the pipette from the pipette holder and attach and prime the PNT pipette before attempting PNT (Please refer to previous materials and methods section chapter 5 or full PNT method).

5.3.2.2 Polar body removal performed in the absence of cytoskeletal inhibitors followed by PNT

PNT was attempted on a number of zygotes (n=19) which had undergone polar body removal but in un-supplemented G1v5plus media (media lacking cytoskeletal inhibitors). Polar bodies were therefore removed in G1v5plus media before they were transferred to another drop within the same manipulation dish containing media supplemented with the cytoskeletal inhibitors. The zygotes were then incubated in the drop containing the supplemented media for between 10-15 minutes prior to attempting PNT. This allowed time to take off the zona drilling pipette and replace it with the PNT pipette. However this did increase the time taken to perform the entire procedure, given to the time required to move the zygote between drops of media in the manipulation dish, change biopsy pipettes, primer biopsy pipettes and orientate the zygote so as to find the hole originally made to remove the polar bodies. For those zygotes which polar bodies were successfully removed in G1v5plus media, PNT was subsequently attempted a significant number survived both procedures and demonstrated potential for onwards development (n=14/19).

5.3.2.3 Polar body removal performed in media supplemented with cytoskeletal inhibitors immediately prior to performing PNT

Those zygotes for which polar bodies were successfully removed in the presence of cytoskeletal inhibitors (n=10) were also used to attempt PNT. Here the zona drilling pipette used to remove the polar bodies was taken off the pipette holder and the PNT pipette attached in its place. It was possible to successfully remove the pronuclei intact from these zygotes (n=8). However we did find that those zygotes which had previously undergone polar body removal in the presence of cytoskeletal inhibitors were more ‘leaky’ which we believe was due to the prolonged exposure to the cytoskeletal inhibitors. Two zygotes which lost a considerable amount of cytoplasm did go on to degenerate.

5.3.3 Issues associated with performing polar body removal and PNT in the presence/absence of cytoskeletal inhibitors

Initial experiments performed to examine the feasibility of polar body removal had primarily been performed in G1v5plus media, with initial results proving encouraging, although on several occasions the zona drilling pipette used to remove the polar bodies inadvertently breached the cytoplasm of the zygote, which usually resulted in the zygote degenerating during the recovery period (Fig5.55b). Also due to the limited time that pronuclei remain visible for following fertilization until pronuclear breakdown (~17-20hrs) it is essential to maximise the time and ensure pronuclear transfer is performed and completed before the pronuclei breakdown due to the limited number of abnormally fertilised zygotes often received.

Therefore in order to minimise these issues experiments were performed where the zygotes were incubated in the cytoskeletal inhibitors prior to performing polar body biopsy. This it was hoped would prevent issues surrounding lysing of the zygote, if the cytoskeleton was relaxed and would also allow for PNT to be performed immediately following polar body removal without then need to transfer the zygote between different drops of media within the manipulation dish.

However due to the significantly reduced size of the zona drilling pipette (10µm) used to remove the polar bodies in comparison to the PNT biopsy pipette (28µm) and also issues regarding the air syringe controlling suction on the biopsy pipette, there was a significant lack of fine control during PB removal. Indeed on several occasions when removing the polar bodies from zygotes incubated in cytoskeletal inhibitors (even following short periods of incubation in the cytoskeletal inhibitors <5mins) the polar bodies would often rush out from the perivitelline space and into the biopsy pipette, immediately followed by the cytoplasm of the zygote, resulting in lysing and degeneration of the zygote.

For those zygotes which had successfully undergone polar body removal, time was able to be saved as both PB removal and PNT procedures were able to be performed in the same media. However the presence of the cytoskeletal inhibitors did make the polar body removals more difficult to remove and consequently often took more time. Therefore it appears possible to remove the polar bodies in the presence of media

supplemented with cytoskeletal inhibitors however it does also introduce additional complications not encountered when removing the polar bodies in non-supplemented G1v5plus media.

	Overall number	Number degenerating during procedure	Number surviving procedure
Polar bodies removed in G1v5plus culture media only	29	2	27
Polar bodies removed in cytoskeletal inhibitors and used for PNT	10	2	8
Polar bodies removed in the absence of cytoskeletal inhibitors and used for PNT	19	5	14

Table: 5.1 abnormally fertilised human zygotes used for polar body removal ad PNT

Above table details the number of zygotes which were initially underwent polar body biopsy and later PNT. Here the polar bodies were removed for a number of abnormally fertilized human zygotes (n=39). The polar bodies were removed in media which was (n=10) and was not supplemented with cytoskeletal inhibitors (n=19) and PNT attempted.

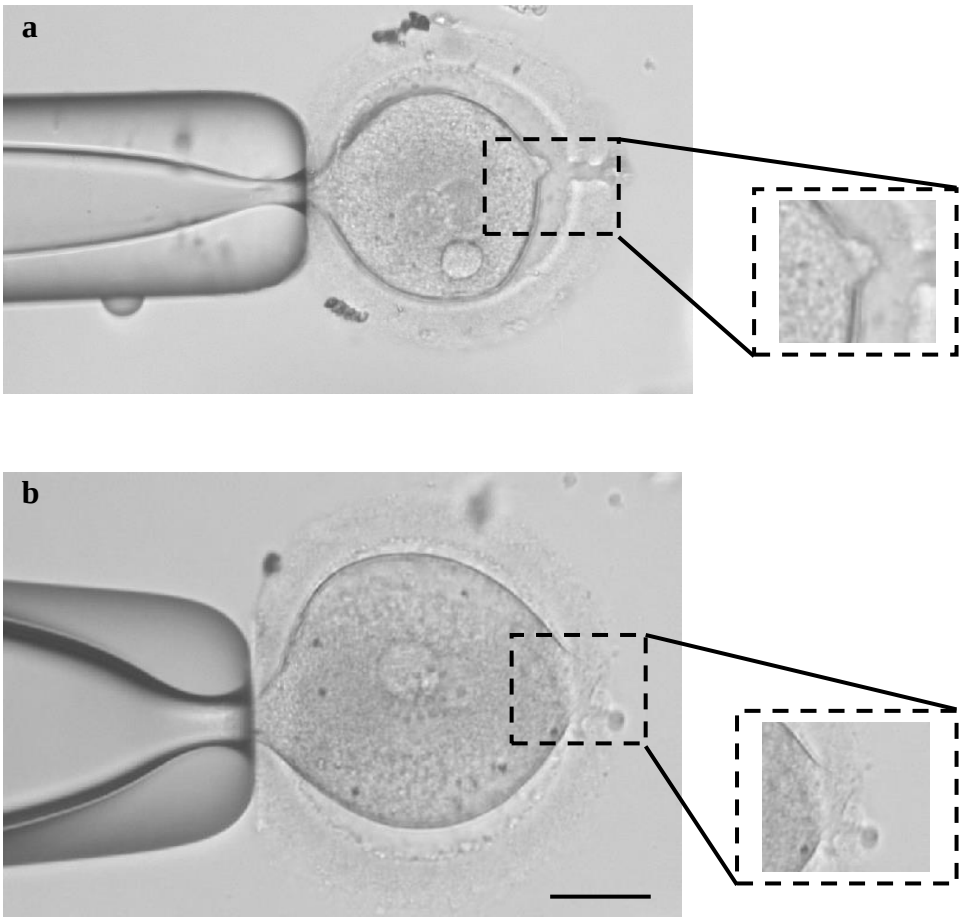


Figure5. 5 Removal of polar bodies prior to performing pronuclear transfer in the presence and absence of cytoskeletal inhibitors

Above images represent abnormally fertilised human zygotes which were incubated in media containing cytoskeletal inhibitors prior to removing the polar bodies. (a) Here we found that this relaxed the cytoplasm to such an extent that during polar body removal often a small amount of cytoplasm was also removed with the polar body. However if the ooplasmic membrane was 'nicked' with the polar body biopsy pipette during the biopsy procedure if the cytoskeletal inhibitors were present the zygote did most often not go on to degenerate. (b) We did attempt to remove the polar bodies when the zygote was placed in media without cytoskeletal inhibitors, although when the ooplasmic membrane was breached accidently the zygote often went on to lyse. Scale bar, 50μm.

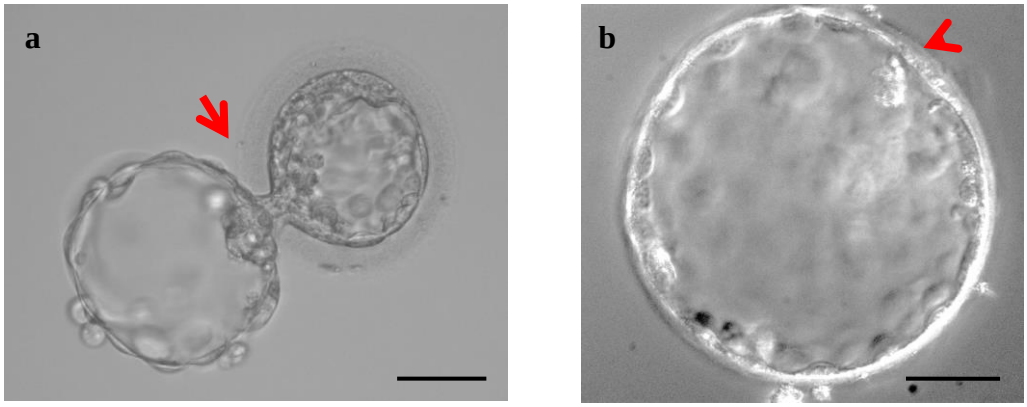


Figure5. 6 Development following polar body biopsy

(a)Example of a manipulated (polar body biopsied zygote), which successfully developed to blastocyst stage; hatching through the hole made to allow the biopsy pipette to enter the zygote (as indicated by arrow) (b) Cultured, unmanipulated control embryo which successfully developed to a blastocyst stage embryo, here the zona pellucida thinned naturally. Scale bars, 50µm.

5.4 Development following polar body biopsy

Here we biopsied a total of n=39 abnormally fertilised human zygotes, a significant number (n=27) of which successfully survived the biopsy process and went onto cleave and develop *in vitro*. (Note - all zygotes which did not survive the polar body removal procedure were not included in data analysis). Here development was good in those biopsied zygotes, with 14% (n=4) (Fig5.7) developing to the blastocyst stage *in vitro*. Development was comparable to data collected for unmanipulated cultured embryos, where development to blastocyst rate was 16% (n=10) (Fig5.7 and Table 5.2).

Due to issues surrounding premature hatching due to the presence of artificially made holes in the zona pellucida so as to allow the biopsy pipette to gain entry through the zona pellucida. Immuno-labelling experiments were performed so as to determine if cell number and the integrity of the trophectoderm and ICM was affected by the hole and biopsy process in comparison to non-biopsied cultured control embryos.

5.5 Immuno-labelling of polar body biopsied embryos

Those zygotes which successfully developed to blastocyst stage embryos were fixed (day 5-7) in 4% PFA for 30mins. These were then used in immuno-labelling experiments using DAPI to examine cell number and Nanog to examine the number of pluripotent cells in relation to the inner cell mass (ICM). Cell numbers determined for those zygotes (n=4) which underwent polar body biopsy and successfully developed to blastocyst stage embryos (~79) were not found to differ significantly ($p=0.7$) to those of the unmanipulated cultured only (n=10) control embryos (~90). The number of Nanog positive cells was also found to be similar between the polar body biopsied (~8) and control blastocysts (~10) (Fig5.9).

	<four-cell	Four-cell to eight-cell stage	>Eight-cell stage
Polar body biopsied zygotes	11% (n=3/27)	15% (n=4/27)	74% (n=20/27)
Unmanipulated control zygotes	15%(n=7/48)	15%(n=7/48)	70% (n=34/48)

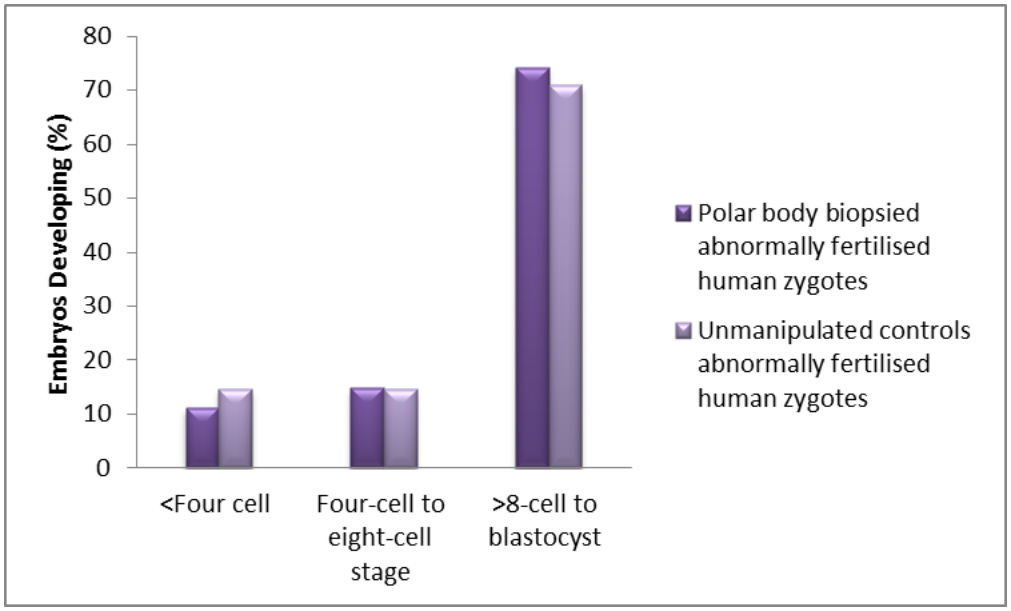


Figure5. 7 and Table5.2 Development of polar body biopsied versus unmanipulated control abnormally fertilised human zygotes

Above graph represents development attained for those zygotes which underwent polar body biopsy (n=27) (dark purple bars), versus development observed in unmanipulated control (cultured only) (n=48) (light purple bars) abnormally fertilised human zygotes. Table representing developmental stage attained by embryos undergoing polar body biopsy versus unmanipulated controls.

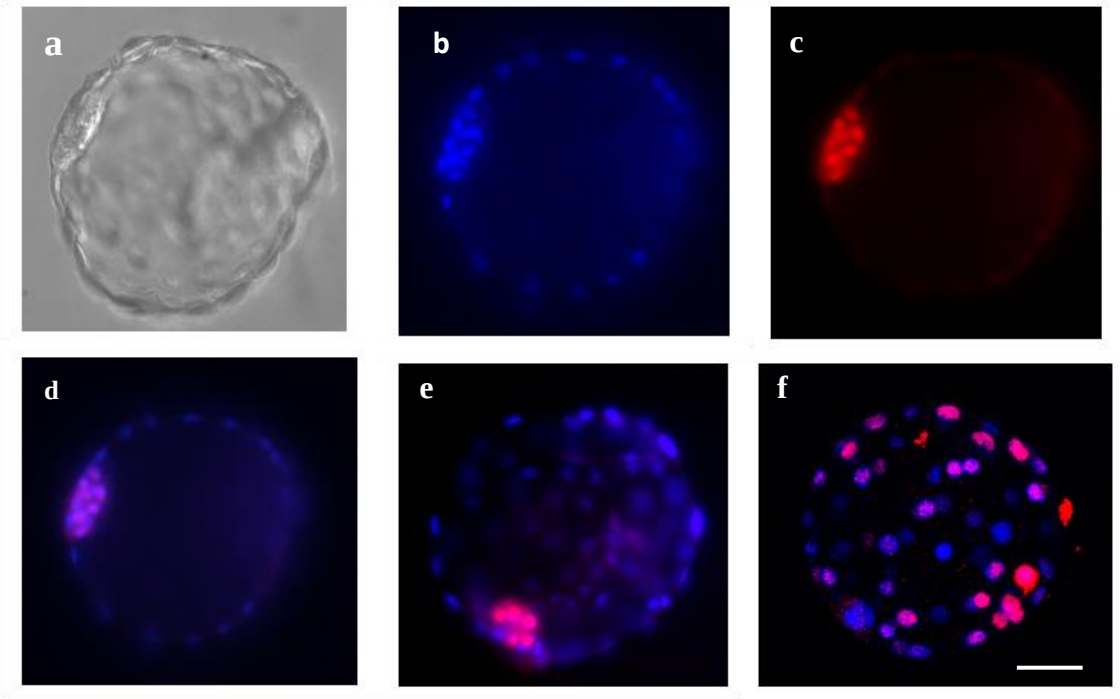


Figure5. 8 Analysis of polar body biopsied and control blastocysts

Blastocysts were fixed in 4% PFA for 15-30 minutes and then stored in PBS until staining. Blastocysts were stained for DAPI (blue) Nanog (red) (all images taken at x20 magnification). Images (a-d) represent the same blastocyst stained for DAPI (b) and Nanog (c) which were then overlaid in order to determine the number of co-expressing DAPI and Nanog cells (e). From the DIC image there is a clear and distinct inner cell mass (ICM) present which contains a distinct population of Nanog expressing cells. However not all abnormally fertilized embryos exhibited restricted patterns of Nanog expression (image f). Scale bars, 50µm.

	Total cell counts (DAPI)	Nanog positive cells
Polar body biopsied embryos(Manipulated)	79	8
Control -cultured only embryos(Unmanipulated)	90	10

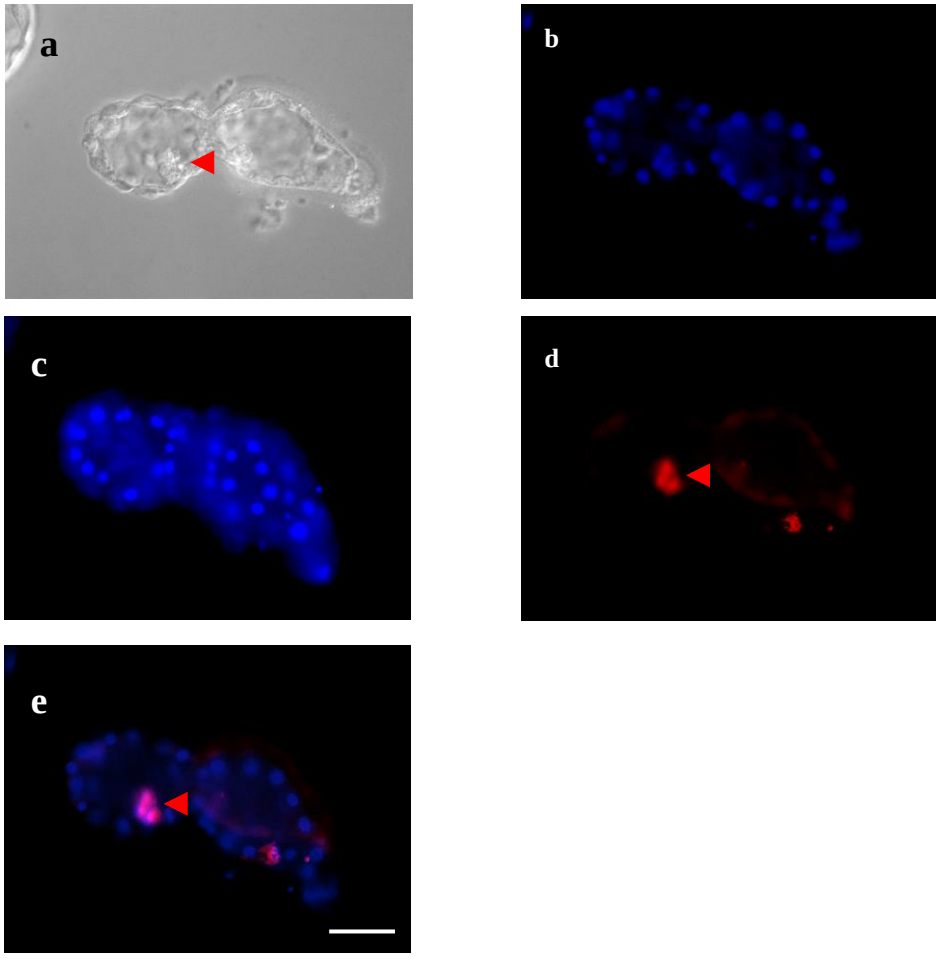


Figure5. 9 and Table5.3 Images represent abnormally fertilised human zygotes, which had a small hole made in the *zona pellucida* (ZP) to allow for polar body biopsy.

Blastocyst stained for DAPI (blue) Nanog (red) (all images taken at x20 magnification). DIC image of blastocyst (a) the same blastocyst stained for DAPI (b and d) Nanog (d) and then images acquired from staining for DAPI and Nanog were then overlaid in order to determine the number of co-expressing cells in different parts of the blastocyst (e). From the DIC image there is a clear and distinct inner cell mass (ICM) present (a) (as indicated by arrow) which contains a distinct population of Nanog expressing cells (d and e) (as indicated by arrow). Scale bars, 50µm.

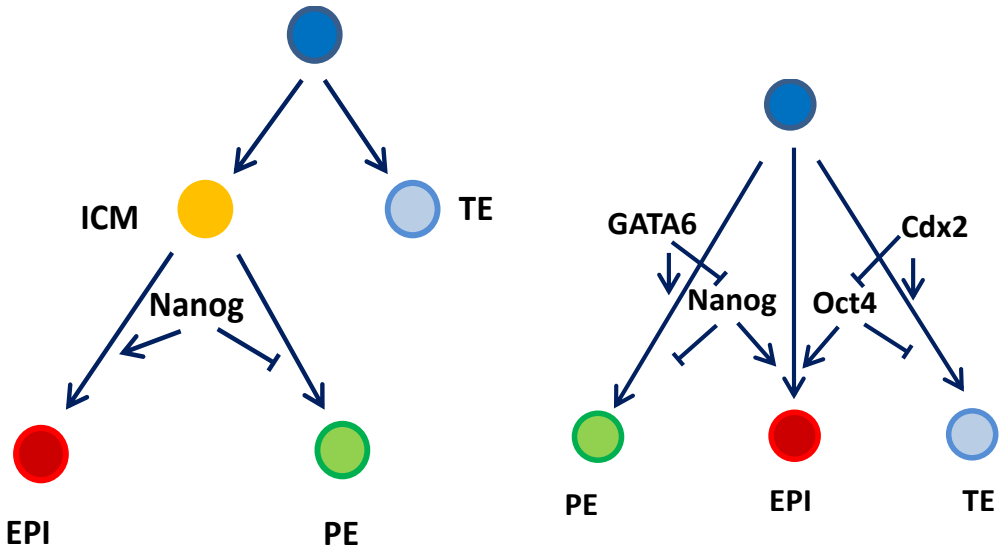


Figure5. 10 Progressive segregation of blastocyst lineages

Blastomeres first become specified to form the inner cell mass (ICM) or trophectoderm (TE), dependent upon the level of Oct 4 expression. Oct4 promotes ICM formation and inhibits TE development. ICM cells then become specified to be epiblast (EPI) or primitive endoderm (PE) dependent upon the levels of Nanog, with Nanog acting to promote EPI and block PE development. (B) The choice of becoming EPI, PE or TE is not progressive, but depends on localized expression of both positive and negative factors. Oct4 and Nanog together promote EPI formation, with Oct4 inhibiting TE formation and Nanog inhibiting PE formation. Whilst Cdx2 promotes TE formation and inhibits EPI formation; GATA6 promotes PE formation and blocks EPI formation.

5.6 Discussion

In this chapter I examined the feasibility of polar body removal. This was because of the concern that in the presence of the fusing agent HVJ-E, polar bodies could fuse back with the zygote in so altering the chromosomal constitution of the zygote. HVJ-E is introduced into the zygote following re-introduction of the pronuclear karyoplasts into the (donor) recipient zygote. If enough HVJ-E is introduced and persists within the perivitelline space, following karyoplast fusion, this could theoretically allow the polar bodies to re-fuse back with the zygote.

Polar bodies are bi-products of the female meiosis and not directly involved in embryo development, therefore they can be safely removed through biopsy procedures (Mastenbroek et al, 2007; Kuliev and Rechitsky 2011; Levin et al, 2011). These structures are not maintained throughout preimplantation and degrade shortly after formation, usually within 17–24 hr, and the resulting fragments remain entrapped within the zona pellucida (Longo, 1997; Ebner et al, 2000). Therefore, due to the transient nature of polar bodies it is very unlikely that they would be able to fuse back with the zygote, even in the presence of fusing agents. However given the concern which had been raised surrounding the potential for this to occur, it was important to address the problem and therefore assess the feasibility of polar body removal prior to performing the PNT procedure.

Traditionally the PNT procedure is performed using media supplemented with cytoskeletal inhibitors (Cytochalasin B and nocodazole) (Craven et al, 2010). The addition of these cytoskeletal inhibitors to routine culture media, relaxes the cytoskeleton allowing for the safe and successful removal of pronuclei. As polar body removal was ideally performed prior to performing PNT, I set out to examine the feasibility of removing the first and second polar body in media supplemented with the two cytoskeletal inhibitors. However I also compared this to removing polar bodies in un-supplemented media (without the cytoskeletal inhibitors), to determine if this impacted upon the removal of the polar bodies. Ideally by removing the polar bodies in media supplemented with the cytoskeletal inhibitors then zygotes could be used immediately to perform PNT, without the need to move the zygotes between different types of media.

The first approach examined removing polar bodies in media supplemented with cytoskeletal inhibitors. Removing the polar bodies in the presence of cytoskeletal inhibitors, did prove to slightly complicate the procedure. This was believed to be due to the relaxed nature of the cytoskeleton which meant that when removing polar bodies which resided in close proximity to the cytoplasm of the zygote there was a substantially increased risk of damaging the cytoplasm of the zygote. Also by removing polar bodies in media supplemented with cytoskeletal inhibitors the length of time embryos were exposed to these chemicals was increased. This is a concern as cytoskeletal inhibitors have been shown to demonstrate cell toxicity following extended exposure. However brief periods of exposure to cytoskeletal inhibitors at low concentrations (within the range of 2.5-10μM) has been shown does not perturb development (Otaegui et al, 2005). Therefore provided that zygotes are not left within the media supplemented with cytoskeletal inhibitors for extensive periods (>9hours) no long term affects should be observed.

The removal of polar bodies from zygotes using un-supplemented media, I found removed the risk of the cytoplasm of the zygote being removed during the polar body removal. However as the cytoskeletal inhibitors were not present during this set of manipulations, if by chance we did 'nick' the cytoplasm of the zygote, the zygote was often unable to recover and degenerated as a result. This was often due a lack of malleability of the zygote in the absence of the cytoskeletal inhibitors but also due to the presence of connections which have been shown to exist between the polar bodies and the cytoplasm of the zygote (Montag et al, 2012). In order to remove the polar bodies successfully these 'ties' had to be broken, which often resulted in breaching of the zygote cytoplasm and the zygote degenerating.

As zygotes were to be used for PNT experiments following polar body removal, this influenced decisions taken when removing polar bodies, including, the most optimal position to make a hole to perform the biopsy procedure. The idea was that the same hole made to remove the polar bodies could then be re-used to remove the pronuclei. However the position of the polar bodies within the perivitelline space did not always allow for this to be accommodated. For example in zygotes produced following intracytoplasmic sperm injection (ICSI), the position of the first and second polar body differed. This is due to the fact that when MII stage oocytes are fertilized using the ICSI procedure the oocyte is orientated so that the polar bodies are located at either the

6 or 12 o'clock position to ensure that the spindle is not damaged as a result of the injection process. This is different to the situation following routine IVF insemination, where first and second polar bodies can be found within a close proximity. The position of the polar bodies in ICSI zygotes can often differ by 45^{o}. This therefore made it difficult to remove the first and second polar body from the ICSI fertilized zygotes, firstly using the same hole and secondly taking care as to not damage the zygote. However this study did make use of abnormally fertilized human zygotes and therefore the location of the polar bodies may have been influenced due to their abnormal fertilization.

The polar body biopsy (PBB) procedure is said to be one of the safest forms of biopsy to perform during embryonic development. Indeed where possible PBB is now performed in place of conventional cleavage stage PGD in clinical practice due to the minimal invasive nature of the procedure (Verlinsky et al, 1990). Therefore the impact of the PBB procedure upon development, embryo quality and viability is often regarded as minimal (Munne et al, 1999; Magli et al, 2004; Wells et al, 2002). However given the complications I encountered when performing polar body it was important to ascertain that development was not affected by the polar body removal procedure. I therefore examined development in embryos which had undergone polar body biopsy. The blastocyst development rate observed for PBB embryos was comparable to that attained by unmanipulated cultured control embryos (14% versus 16%, respectively).

There is further evidence that as the degree of fragmentation found within the perivitelline space increases embryo quality is reduced (Ebner et al, 2000). Indeed I found that development observed for embryos following polar body removal and any further residual fragmentation did appear to help enhance embryo developmental potential. Therefore by removing the polar bodies and if possible any additional fragmentation present at the same site we may not only be eliminating the potential threat posed by fusing back of the polar bodies with the zygote due to HVJ-E mediated fusion but also helping to improve developmental potential and the overall embryo quality.

There are other studies which have reported a reduction in development for embryos which underwent PBB. One such study examining the effects of PBB reported the unfavourable effect it had on subsequent embryo development in terms of worse

cleavage patterns, higher fragmentation rates, and lower number of blastomeres (Levin et al, 2011). However this study did examine the potential of first and second polar body removal and also highlighted the fact that when they initially started gathering data they had less experience in the technique (<100 cycles). This 'learning curve' they said could have impacted upon the outcomes of the study. They also commented on the effect of biopsying at the egg stage rather than the zygote stage alone may have impacted on future embryo development due to the additional intervention. For example it is common practice to biopsy both polar bodies at the same time at the zygote stage.

However the method used to perform PBB can differ between laboratories; this could therefore influence outcomes including developmental potential following PBB. The two main techniques used to perform PBB are laser-assisted and partial zona dissection. Although the effect the biopsy technique alone has on subsequent embryo development and quality is not always easy to assess, due to the numerous other variables which cannot always be controlled for, including; embryo handling, exposure to non-natural light sources, culture media and also quality of the eggs/embryos.

However the use of laser ablation of the *zona pellucida*, has been suggested could impact on embryo development potential. Despite evidence to suggest the use of laser ablation minimum results in a minimal amount of heat dissipation, which is aided by the use of 'isotherm rings' it cannot always be guaranteed. Indeed a study published in 2011, investigated whether the viability of blastocysts developed from laser- versus partial zona dissection treated 1PN oocytes was negatively affected by the method of biopsy, which they evaluated by comparing their total cell number (TCN). Interestingly they found that survival and development was not affected as a direct result of the technique used to make the opening in the *zona pellucida.* However the TCN of laser-derived blastocysts was significantly lower than the TCN of blastocysts developed from control and PZD 1PN oocytes. They concluded therefore that viability of blastocysts developed from laser-treated 1PN oocytes was reduced as a result of this particular method of zona dissection. However my own analysis revealed no significant difference (p=<0.7) in the overall cell number for those zygotes which underwent polar body biopsy (~79 cells) and those unmanipulated controls (~90 cells).

Although the study by Macas (2011) determined that blastocyst viability was reduced as a direct result of laser-assisted dissection of the *zona pellucida*. It is difficult to see how the exact technique used to make the opening in the *zona pellucida* can be distinguished from the effect of the 'size of the opening' made in the zona pellucida and its impact on blastocyst viability. Indeed artificially breaching the zona pellucida has been reported to alter the normal thinning process of the zona pellucida directly (shown to hatch through a thicker zona pellucida, while having a smaller embryo size) increasing the risk of premature hatching at the 'early blastocyst stage' (~day5) (A De (Malter and Cohen, 1989; Montag *et al.*, 2000; Schmoll *et al.*, 2003; Vos *et al*, 2008; Kirkegaard et al, 2011). Premature hatching is not often observed during the normal hatching process. This is because the *zona pellucida* of non-manipulated embryos demonstrate a unique 'elastic' like property which allows the zona pellucida to expand as the blastocyst begins to expand, with the development of the blastocoels cavity. This is in turn associated with thinning of the zona during what is referred to as the 'contraction cycle' (Cohen et al, 1991).

It is indeed more likely that the premature hatching-like process observed following partial zona dissection would explain the differences observed in TCN. Blastocysts developed from the artificially breached embryos initiating a hatching-like process, with cells escaping from the large hole in the zona on the morning of day 5 or even earlier (Cohen, 1991; Macas et al, 2011; Kirkegaard et al, 2011). Studies performed to examine TCN in blastocysts determined to have initiated the hatching process found, no further increase in TCN following initiation of the process (Fong and Bongso 1998) . This has led to the suggestion that mitotic activity is suspended and can only be triggered again if such blastocysts attach and embed among endometrial cells (Macas et al, 2011). This may suggest that artificial breaching of the zona pellucida regardless of the technique used negatively impacts upon the mitotic activity of the cells forming these blastocysts. An alternative explanation has also been suggested, i.e. that the metabolic requirements may be inadequate at the time of hatching and that this can promote the process of cell death, which *per se* may also reduce the TCN. Since the premature hatching observed in the present study mimics in some ways the mode of blastocyst hatching *in vitro*, it is concluded that a similar mechanism must also be involved in the decrease of the TCN of the laser-derived group of blastocysts (Macas et al, 2011). Indeed it is possible to perform PBB using specially designed spiked pipettes, known as

'zona drilling' pipettes. It is often safer and easier to perform PBB with the aid of a hole in the zona pellucida.

Although it appears that artificial breaching of the *zona pellucida* does not appear to affect the overall survival and ability of embryos to successfully develop to the blastocyst stage when investigated further the viability of these same blastocysts maybe irreversibly impaired (Cieslak-Janzen, et al 2006; Magli et al, 2004; Macas et al, 2011). Furthermore blastocysts hatching from artificially made biopsy holes could damage the integrity of the trophoblast and the ICM through expulsion through a narrow opening due to the fragile nature of the human blastocyst (Cohen et al, 1991).

It is therefore important that blastocysts developing from embryos which have had artificial openings made in their *zona pellucida* are examined to determine that lineage specification is not perturbed in light of the differences observed in TCN (Macas et al, 2011). There are several key factors which have been identified to be essential for correct cell lineage specification during embryogenesis (Rossant, 2004). One such factor is Nanog which is responsible for maintaining cells in a pluripotent state. Nanog expression gradually becomes restricted to the ICM by the blastocyst stage (~day 5), where it is then maintained in the epiblast of the pre-gastrulation embryo until formation of the primitive germ cells, before it eventually becomes restricted to the germline (Palmieri et al, 1994; Rossant, 2004). Nanog expression was examined in both manipulated and unmanipulated (control) embryos in those embryos which successfully developed to blastocysts. For those blastocysts which contained a distinct ICM (not all did) we found there to be sufficient down regulation of Nanog in the trophectoderm cells compared to the ICM population of cells, with a distinct population of Nanog cells expressed in the ICM alone Importantly no significant difference was observed in the number of Nanog positive cells we identified in the polar body biopsied blastocysts (~8) versus the unmanipulated controls (~10). Despite the relative high cell numbers observed for these blastocysts, it is worth while remembering that these experiments were performed in abnormally fertilised human zygotes. It has already been demonstrated that developmental potential is reduced in abnormally fertilized embryos and therefore it is likely that cell specification is perturbed for these embryos due to their abnormal chromosomal constitution (Feenan and Herbert, 2006).

These studies have therefore demonstrated that development to the blastocyst stage *in vitro* following polar body biopsy does not negatively impact upon development or indeed lineage restriction and specification. Polar body biopsy does appear feasible although it does increases the length of time required to perform the PNT procedure which is already lengthy and performed against the clock due to the limited period of time pronuclei remain visible for prior to syngamy and pronuclear breakdown. However with the use of a double pipette holder time can be saved and its feasibility boosted. By removing the polar bodies the threat of possible fusion due to the use of HVJ-E would be completely eliminated. Therefore not only helping improve the developmental capacity able to be attained but also the overall embryo quality, which may help improve development rates achievable following PNT.

Chapter 6

Vitrification

Chapter 6 Vitrification

6.1 Introduction

Since the first successful reported pregnancy from a frozen embryo in 1983 (Trounson and Mohr, 1983), embryo cryopreservation has become regarded as an integral part of the IVF process allowing couples to store excess embryos for future treatment (Trounson and Mohr, 1983; Valojerdi et al, 2009), thus maximising the chance of a pregnancy from each round of ovarian stimulation (Loutradi et al, 2007).

Until recently the most commonly used approach to cryopreserve embryos within IVF units globally has been slow freezing or which is sometimes referred to as controlled rate freezing. This utilises programmable freezers to control the rate of cooling in a step-wise manner, over several hours. However more recently, the technique known as vitrification has begun to replace traditional slow freezing programmes within clinical IVF units. The word to 'vitrify' literally means to turn to a glass-like state. In order to achive this, vitrification requires very rapid (high) cooling rates, which in so substantially minimize the risk of ice crystals forming inside cells. Together with the high cooling rate, vitrification also requires the inclusion of cryoprotectants within its vitrification solutions in order to ensure cells are dehydrated sufficiently prior to plunging in liquid nitrogen. These core principles help promote improved embryo survival compared with conventional controlled-rate freezing (L.Hyslop unpublished data).

In the following chapter I aim to address and explain the main principles involved in establishing successful cryopreservation programmes. So as to set the scene in light of the work I have performed to examine the potential of vitrification in relevance to the nuclear transfer technique, pronuclear transfer for the purposes of preventing the transmission of mtDNA disease.

6.1.1 Controlled rate freezing

Controlled rate freezing involves the dehydration of embryos by exposing them to increasing concentrations of cryoprotectants followed by a mechanically controlled step-wise decrease in temperature. Controlled rate freezing is performed over several hours and therefore viewed as an extremely time consuming process and also requiring accurately controlled and expensive programmable freezing equipment. Despite this, controlled rate freezing has conventionally proven the preferred option for embryo cryopreservation, primarily due to the relatively low concentrations of cryoprotectants contained in the freezing solutions (Kolibianakis et al, 2009). However, while the low concentrations of cryoprotectants used for controlled rate freezing limit the potential toxic and osmotic damage to embryos, they are potentially insufficient to prevent intracellular ice crystal formation, which can lead to cell damage and developmental arrest (Bryant, 1995; Shaw and Jones, 2003).

In general, cryoprotectants act to lower the cell's freezing point by osmotically permeating through the cell membranes and slowly replacing the intracellular water (Shaw and Jones, 2003; Loutardi et al, 2007). However a fine balance exists between the rate of water loss from a cell and extracellular ice crystal formation, as excessive dehydration can increase the intracellular concentrations of cryoprotectants to increase to potentially toxic levels (Shaw and Jones, 2003; Lane et al, 1999; Liebermann et al, 2002; Loutardi et al, 2008). Therefore although the concentrations of cryoprotectants within the controlled rate freezing solutions are relatively low compared to those found within vitrification solutions the total time period over which embryonic samples are exposed to these solutions does

6.1.2 Vitrification

Vitrification, literally meaning 'to turn to glass', is frequently referred to as a novel approach to embryo cryopreservation. However vitrification has been used as an alternative to controlled-rate freezing' for the past decade with significantly improved results (Balaban *et al*, 2008). Indeed Rall and Fahy were the first to report on the successful vitrification of mammalian embryos back in 1985, with the first reported pregnancy from a vitrified embryo following in 1990 (Valojerdi *et al*, 2009). Another

concern which has delayed the implementation of vitrification within IVF laboratories is potential for compromising sterility of embryonic samples due to the requirement for direct exposure to liquid nitrogen, key to ensuring successful vitrification (Bielanski and Vajta, 2009). However significant improvements have been made in the development of vitrification devices and solutions, many formulated specifically for the different stages of embryonic development which has led to the technique replacing controlled rate freezing within the majority of IVF units worldwide.

There are several key principles to ensure efficient and successful vitrification performance. This includes firstly the inclusion of cryoprotectants at high concentrations in the final vitrification solutions, secondly prompt and accurate direct contact with liquid nitrogen and thirdly highly skilled operators. By ensuring these principles are adhered to the outcomes achievable with vitrification are second to none and most importantly promise significantly improved survival outcomes compared to conventional controlled rate freezing.

There are two types of cryoprotectants included within vitrification solutions 'permeating' and 'non-permeating'. As the names suggest the 'permeating cryoprotectants' (ethylene glycol, propanediol and DMSO) are able to enter the cell and replace water molecules inside the cells, thereby preventing the formation of intracellular ice formation. Whilst the 'non-permeating' (sugars e.g. sucrose) cryoprotectants are macromolecules which remain outside the cell acting to increase the extracellular osmolarity and in aid in dehydrating the cell. Therefore the dual action of 'permeating' and 'non-permeating' cryprotectants allows them to act like 'antifreeze' whereby they reduce the freezing temperature (due to the elimination of water) and increase the viscosity (Bouvet and Ben, 2003). Furthermore by ensuring the sufficient elimination of water from the cells the risk posed by potential ice crystal formation is significantly reduced. This is important as the induction of cryoinjuries can considerably reduce survival upon warming (Loutardi et al, 2008).

6.1.3 Principles of vitrification

6.1.3.1 The role of cryoprotectants

A cryoprotectant is defined as a substance that is used to protect biological tissue from freezing damage. There are two types of cryoprotectants included within vitrification solutions 'permeating' and 'non-permeating'. As the names suggest the 'permeating cryoprotectants' (ethylene glycol, propanediol and DMSO) are able to enter the cell and replace water molecules inside the cells, thereby preventing the formation of intracellular ice formation (Kasai, 1996). Whilst the 'non-permeating' (sugars e.g. sucrose) cryoprotectants are macromolecules which remain outside the cell acting to increase the extracellular osmolarity and in assist in dehydrating the cell. Therefore the dual action of cryprotectants allows them to act like 'antifreeze' whereby they reduce the freezing temperature (due to the elimination of water) and increase the viscosity. Furthermore by ensuring the sufficient elimination of water from the cells the risk posed by potential ice crystal formation is significantly reduced. This is important as the induction of cryoinjuries can considerably reduce survival upon warming (Loutardi et al, 2007). The role of cryoprotectants in eliminating water is also key to lowering the freezing point and subsequently allowing the cooling rates of >25,000^{o}C/min to be achieved (Isachenko et al, 2005; Yavin et al, 2009; Zhang et al, 2011).

However the cryoprotectant often proves the predominate source of toxicity within vitrification (Kasai et al, 1992a, 1996). Although by opting for cryoprotectants with low toxicity and good permeation properties toxicity can be minimized. Ethylene glycol is commonly used as the preferred cryoprotectant due to its relatively low toxicity and low molecular weight giving it a high permeation ability (Gilmore et al, 1995; Newton et al, 1998; Sommerfield and Niemann, 1999; Emiliani et al, 2000). In order to further minimize the effect cryoprotectants exert on cells a combination of cryoprotectants is generally used, with the most common combination proving a mix of ethylene glycol (ETOH) and propanediol (PrOH). By using a combination the concentration at which each of the cryoprotectants are included at within the vitrification solutions can be halved and the risk of toxicity posed to cells significantly reduced. Further steps have been taken to reduce the risk further, by including an equilibration step prior to a final vitrification step. The equilibration solution generally contains the cryoprotectants (both 'permeating' and 'non-permeating') at half of the concentration at which they are present at within the final vitrification solution. Cells are therefore able to be left longer in the initial equilibration solutions before they are exposed to the final vitrification solution. Indeed the introduction of multiple step

protocols for the vitrification of oocytes and embryos has proven to be one of the key factors in revolutionizing the vitrification procedure.

6.1.3.2 Duration of exposure to cryoprotectant

Exposure times are usually reduced when the cryoprotectant concentration is high and increased when the cryoprotectant concentration is low. The most commonly used strategy adopted to avoid toxicity associated with vitrification solutions is to therefore to limit the duration of exposure by oocytes/embryos to the vitrification solutions (Kasai, 1996). However insufficient exposure to the cryoprotectants can result in incomplete permeation and intracellular ice crystal formation, which cause damage upon thawing; whilst over exposure to cryoprotectants can result in toxic injury (Yang *et al*, 2007).. Therefore a compromise is often made between duration of exposure and concentration of cryoprotectants, in order to achieve the best outcomes possible. Often the deciding factor defining optimal duration of exposure often rests upon the stage of embryonic development the sample is vitrified at (Kasai, 1996).

6.1.3.3 Single versus multiple steps

The number of steps included in a vitrification protocol is also a defining factor when choosing a vitrification system. Single exposure vitrification solutions may not only allow insufficient time for adequate penetration of the cryoprotectants but also expose the cells to undue toxic side-affects. The use of two-step protocols overcomes this issue allowing cells to be equilibrated in initial 'equilibration solutions' for longer periods (up to 15 minutes) containing the cryoprotectants at lower concentrations before a short exposure to a second 'vitrification solution' containing the cryoprotectants at a higher concentration (Liebermann et al, 2002; Kader et al, 2009). Allowing the cryoprotectants to more gradually and effectively penetrate the embryo; whilst minimizing the risk of osmotic shock and toxicity (Kader et al, 2009).

6.1.3.4 Vitrification devices

During the past decade there has been a significant increase in commercially available vitrification devices, including; the cryotip (Irvine scientific), cryotop (Kitazato), cryolock (Bio-diseno) and cryoleaf (Origio), cryopette (Origio), cryoloop (Vitrolife), Rapid-I (Vitrolife). Vitrification devices normally fall into one of two categories 'open-systems' (direct contact vitrification) or 'closed-systems' (closed system vitrification).

(a) Direct contact vitrification

'Direct contact' or 'open vitrification' systems, have been shown significantly increase the cooling rate, by providing direct contact with the liquid nitrogen interface (24,000^{o}C/min) (Palsz and Mapletoft, 1996). Several studies have demonstrated the excellent survival outcomes within the order of >90% achievable when vitrifying using a 'open vitrification' device (Kuwayama et al, 2007; Liu et al, 2008; Cobo et al, 2008). Indeed 'direct contact liquid nitrogen' was and still is by some considered the 'gold standard' for vitrification. The primary overriding concerns in light of the potential risk of contamination posed by microorganisms able to persist in liquid nitrogen at temperatures as low as -196^{o}C (Bielanski and Vajta, 2009). However in spite of these concerns, so far no disease transmission attributable to this mechanism has been documented in domestic animal or human reproductive biology (Vajta and Nagy, 2006).

(b) Closed system vitrification

Due to concerns regarding the potential risk of contamination posed by micro-organisms 'closed vitrification' systems were developed. However by avoiding direct contact with liquid nitrogen the cooling rate is significantly reduced to ~12,000^{o}C/min almost half of that able to be achieved using 'direct contact' vitrification devices at ~24,000^{o}C/min (E Van Landuyt - 2011). To overcome the reduced cooling rate new vitrification devices have been developed to maximise heat transfer incorporating several key features, including; the use of ultrathin material and ergonomically designed areas to minimise the time taken to load samples.

One such device which has been developed and overcome issues associated with a reduction in cooling rate is the 'Rapid-i™' (Vitrolife) 'closed system' vitrification device. This utilises 'super cooled air' to achieve rapid cooling of sample on submersion in liquid nitrogen, resulting in significantly improved survival outcomes (Larman et al, 2006). The same device has many other unique features, including a

‘50nL’ sized hole located in the tip of the flange of the device to aid in loading of samples in a minimal volume of vitrification media (Figure6.9). The straw into which the loading device resides during storage comes with a metal rod. This metal rod is placed within the outer straw when placed into the box containing liquid nitrogen. The presence of the metal rod acts to create the column of ‘super-cooled air’ inside the straw, into which the vitrification loading device containing the oocyte/embryo is eventually placed. There is now cumulative evidence both published and unpublished which demonstrates the highly efficient nature this device provides when vitrifying, reflected in the survival outcomes (Larman and Gardner, 2011; L. Saragusty and Arav, 2011; Hyslop, unpublished),

6.1.3.5 Operator factors

Vitrification outcome is highly operator dependent and requires a totally different skill set to slow freezing. The embryologist should be rapidly handling the embryos in micro-volumes of highly viscous media. Specific training on the use and storage of the vitrification device should be undertaken prior to commencing vitrification. Furthermore the embryologist should be fully aware of the different critical procedural details that can affect the vitrification outcome. This includes: the types and concentrations of cryoprotectants used and their toxicity threshold, the temperature of the vitrification solution at exposure, the duration of exposure to the final cryoprotectants before plunging into liquid nitrogen, rapid loading, sealing devices (closed-system) and system validation (loading sealing and storage).

6.2 Vitrification at the Pronucleate (PN) Stage

Since in some countries it is illegal to cryopreserve an oocyte after fusion of the pronuclei (PN) due to strictly enforced embryo legislation. It is only possible for those ‘fertilized oocytes’, which have not as yet undergone nuclear fusion (breakdown of pronuclear membranes) so as to regard them as an ‘embryo’ to be cryopreserved. For example, in Germany, the ‘Embryo Protection Act 1990’ grants moral status to those embryos, which ‘nuclear fusion or syngamy has occurred’ (~20hrs p.f.). As a result there has been a great deal of interest in developing a refined method for cryopreserving human pronuclear oocytes (Isachenko et al, 2004).

As a consequence there are is several published reports of successful cryopreservation, at the PN stage using both controlled rate freezing and vitrification (Damario et al, 1999; Park et al, 2000; Isachenko et al, 2005; Orief et al, 2005; Kuwayama, 2007). Many have suggested the PN stage zygote as the optimal stage for cryopreservation (Damario et al, 1999), due to the lack of spindle apparatus, which is suggested accounts for its high post-thaw survival rate (Orief et al, 2005). However as result of the lack of morphological criteria present at the PN stage, grading PN stage zygotes is no easy task, meaning vitrification at the PN stage is not widely used in clinical practice for this reason(Ludwig et al, 2000; Khalili and Mardanian, 2008). However those zygotes which are compatible with development can be assessed upon their ability to undergo the first cleavage division (Orief et al, 2005).

6.3 Oocyte cryopreservation

The clinical interest in gamete cryopreservation has grown significantly during the last decade for which there are several drivers for this. Firstly women undergoing cancer treatments can be rendered infertile by gonadotoxic treatments (Falcone et al, 2004; Gardner et al, 2007). Secondly women are now opting to delay starting their family until later in life and therefore would like the potential option of storing oocytes whilst they are younger (Dondrop et al, 2012). And thirdly oocyte cryopreservation is not as contested ethically as is embryo cryopreservation, making a more favourable stage to cryopreserve at (Gardner et al, 2007).

In contrast to the mammalian embryo, it has proven notoriously difficult to cryopreserve the metaphase II (MII) stage oocyte. One major barrier preventing successful oocyte cryopreservation, being the highly delicate metaphase II spindle, which is highly sensitive to changes in temperature (Chen et al, 2000, 2004; Mullen et al, 2004; Rienzi et al, 2009). Further differences in membrane permeability and cytoplasmic physiological properties render oocytes much more difficult to cryopreserve successfully. One such physiological aspect which has been shown to be altered as a result of cryopreservation procedures is intracellular calcium handling (Gardner et al, 2007). Where there is evidence which suggests that perturbed calcium handling in the

oocyte, can induce premature oocyte activation and zona hardening (Gardner et al, 2007).

Despite relatively early success (Chen, 1986; Van Uem et al, 1987), widespread application of oocyte vitrification was hampered for a considerable time due to the inefficiency of available cryopreservation methods (Oktay et al, 2006). However general improvements and advancements in vitrification devices, solutions and techniques has now made it possible to achieve successful cryopreservation of oocytes (Arav, 1992; Kuwayama et al, 2005; Kuwayama et al, 2007; Ubaldi et al, 2010). Indeed the technology used to perform vitrification has been so well developed and refined that studies conducted examining the efficiency and potential of oocyte vitrification have found no significant differences between fresh and vitrified oocytes (Cobo et al, 2008; Nagy et al, 2009). Indeed a study performed Rienzi et al, (2009) demonstrated that oocyte vitrification followed by ICSI resulted in fertilization and embryo development rates comparable to those observed with fresh oocytes.

6.4 Rationale behind investigating the feasibility and efficiency of vitrification at the metaphase II oocyte and pronucleate stage zygote stages of embryonic development in the context of preventing mitochondrial DNA disease.

The feasibility and potential of the nuclear transfer technique pronuclear transfer (PNT) for preventing the transmission of mtDNA disease has been investigates using abnormally fertilized human zygotes (Craven et al, 2010). The results of these studies published in 2010 successfully demonstrated the compatibility and potential for onward development of those embryos which underwent manipulation. However it is well documented that abnormally fertilized human zygotes demonstrate a limited potential for onward development (Craven et al, 2010). Thus, in order to progress further with the research and better assess development and safety concerns regarding the PNT technique, studies must now be performed using normally fertilised zygotes.

Following the granting of the appropriate licence by the HFEA it is now possible to create embryos, and use those which fertilize normally to assess the PNT technique. Therefore we are now recruiting egg donors, through altruistic egg donor and egg

sharing schemes. However this has presented a logistical problem for several reasons. Ideal PNT transfers are performed using oocytes obtained from two different egg donors. However egg donors do not always exhibit the same response to ovarian stimulation resulting in potential oocyte wastage; either because one of the egg donors fails to respond at all or responds to well, resulting in a potential wastage of oocytes. This is a problem which could also potebtially present itself at a later date, if it becomes possible to offer PNT as a clinical treatment to mtDNA patients. Therefore it is essential that strategies are developed now to allow for cryopreservation of either MII stage oocytes or PN stage zygotes. I therefore set out to investigate the potential of vitrification at the pronucleate and metaphase II oocyte stage of embryonic development. In order to ascertain which would provide the best stage to vitrify at for the purposes of the PNT technique.

Successful cryopreservation strategies are fundamental to the development of IVF-based treatments to prevent transmission of mtDNA disease. Owing to variability between women in their response to ovarian stimulation, it will be very difficult to guarantee that the donor and recipient pairs will be ready to have their oocytes retrieved on the same day. In context of the research and its eventual translation to clinical practice, it will be essential to have reliable methods for cryopreserving oocytes and zygotes, which are compatible with onward development and with the manipulations associated with pronuclear transfer and associated procedures.

6.5 Aims

- To optimise a vitrification protocol for human zygotes
- To determine whether PNT can be successfully performed following vitrification of human zygotes
- To measure mtDNA carryover following PNT between vitrified/warmed human zygotes
- To develop and optimise techniques for vitrification of human oocytes

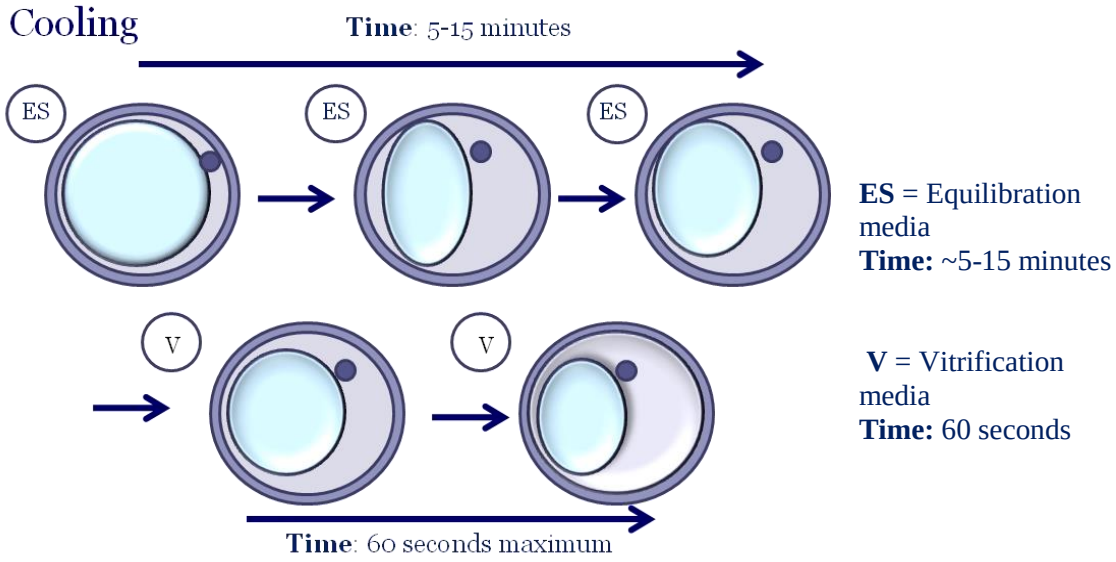


Fig6. 1 Vitrification (cooling)

Above schematic diagram represents the two step process of vitrification (cooling). Here the embryo is initially passed through an equilibration media, which contains the cryprotectants at reduced concentrations (7.5%) ethylene glycol and 1, 2-propanediol. Embryos remain within the equilibration solution until they demonstrate signs of re-expansion (this can vary from 5-15 minutes). Once embryos have re-expanded they are transferred in a minimal volume of equilibration media to the second solution 'vitrification medium'. This contains the cryprotectants at double the concentration (15%, ethylene glycol and 1, 2-propanediol) and also sucrose which acts as a permeating molecule. Embryos should not be exposed to the vitrification solution for longer than 60 seconds, including the time required to load the embryo on to the device and plunge it into liquid nitrogen.

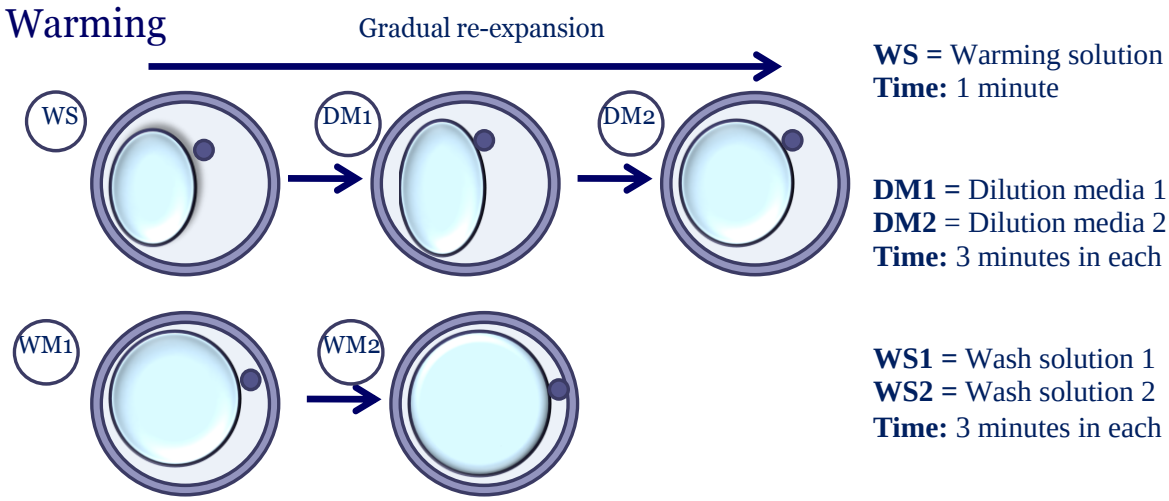


Fig6. 2 Vitrification Warming

Above schematic diagram represents the stages of oocyte/embryo warming, following vitrification. Here the vitrification device containing the embryos is immediately transferred to the initial vitrification warming media following removal from liquid nitrogen storage container (within ~2 seconds). Here embryos remain in the warming solution for up to 1 minute. Embryos are sequentially passed through a series of dilution (dilution media 1 and dilution media 2) (each for a total of 3-minutes) and wash solutions (each for 3-minutes) before they are transferred to an equilibrated culture dish containing G1v5plus media overlaid with ovoil.

6.6 Materials and Methods

6.6.1 Abnormally Fertilised Human Embryos

Abnormally fertilised embryos collected on day 1 of the IVF or ICSI cycle were used in this study. These embryos were identified as abnormal by the presence of either 1 pronucleus (monopronucleate) or 3 pronuclei (tripronucelate) 18-19 hours after insemination. These embryos cannot be used in fertility treatment and are routinely rejected from the IVF program.

6.6.2 Sources of oocytes used in study

Oocytes obtained from follicle reductions (FR), together with IVM and mature metaphase II oocytes which failed to fertilise following IVF/ICSI were used in this study (Failed fertilisation was determined by lack of visible pronuclei on day1 of the IVF or ICSI cycle and failure to cleave by day2). All samples used had previously been consented by patients undergoing fertility treatment at the Newcastle upon Tyne Fertility Centre at Life, following receiving the relevant research project information so allowing them to be used for research purposes.

6.6.3 Vitrification

6.6.3.1 Vitrification using an open vitrification system

(a) Vitrification (cooling)

Equilibration and vitrification solution supplied by Origio (Origio, Medicult, UK) were dispensed into individual wells of four-well nunc dish and left to equilibrate RT for at least 30 minutes prior to use. Using a denudation pipette (vitrolife AB, kungsbacka, Sweden) oocytes/embryos were transferred to Equilibration medium 1 (1ml)(Origio, Medicult, UK) where they remained for 5 minutes before they were transferred to the vitrification medium (1ml)(Origio, Medicult, UK) for <1 minute. Using the Stripper-CC® micropipette oocytes/embryos were immediately loaded onto the tip of the McGill cryoleaf ™ vitrification device in a drop of less than 0.5µl (Origio, Medicult media. Mellehaven 12, 4040, Denmark). The vitrification device containing the embryo was

then plunged directly into liquid nitrogen. Using a pair of tweezers the protective sleeve was pulled down over the tip of the device before the lid of the device was also submerged in liquid nitrogen and the vitrification device placed into the outer cover all performed within the liquid nitrogen phase. All devices were then transferred to pre-labelled cryocaines and transferred to liquid nitrogen tanks for indefinite storage (Fig6.1).

(b) Warming

Vitrification warming solutions supplied by Origio (Origio, Medicult media. Mellehaven 12, 4040, Denmark) were aliqouted into a four-well nunc dish and allowed to equilibrate to RT for at least 30 minutes prior to use. The initial warming solution was placed into a, non-CO_2 incubator and left for minimum of 30 minutes to warm to 37^{o}C. The vitrification carrier was collected from liquid nitrogen (LN_2) storage container and transferred to a portable vessel containing LN_2 until transfer into warming media. The vitrification device was held, so the end of the device containing the zygote remained immersed in the liquid nitrogen whilst the device was removed from its outer cover. Immediately (within 2-3 seconds), the device was transferred to the initial warming medium (1ml, 37^{o}C) ensuring the tip of the vitrification device remained completely submerged in the warming solution until zygotes had been successfully retrieved. Here they remained for a maximum of 1minute. Zygotes were then collected and sequentially incubated in two dilution solutions (1ml) for 3 minutes in each and a further two wash solutions (1ml) each for 3 minutes also (Origio, Medicult media, Mellehaven 12, 4040, Denmark). Zygotes were then transferred to appropriate equilibrated culture medium, where they were allowed time for post-warming recovery (Figure6.2).

6.6.3.2 Vitrification using a closed vitrification system solutions and vitrification device

(a) Vitrification - cooling

Vitrification solutions supplied by (Vitrolife, Gothenburg, Sweden) were used to vitrify zygotes. All vitrification and warming steps were performed at 37^{o}C using 500µl of each solution in a four-well plate (Nunc dish). RapidVit™ is composed of three separate solutions: Rapidvit1, Rapidvit2 and Rapidvit3 (Vitrolife AB, Kungsbacka,

Sweden). Zygotes were initially transferred to a G-mops-like solution (RapidVit1) where they remained for 5-10 minutes. Zygotes were then exposed to an initial solution containing ethylene glycol (RapidVit2) for 2 minutes. The straw with the metal rod was placed inside the SmartBox using the magnet to keep the straw in position. At 1 min 30 seconds a 20µl drop of Vitri3 was made on the bottom of a 35mm falcon dish (BD biosciences, Franklin, USA). At 1 min 50 seconds we began to collect the zygotes (from the RapidVit 2). Zygotes were moved into the 20µl drop of Vitri3 as the 2 minutes elapsed. (Note: the oocytes must be exposed to Vitri3, loaded onto the Rapid-I and placed inside the straw (sitting in liquid nitrogen) within 25-35 seconds). Once the zygotes are in Vitri3 the metal rod was removed from the straw and the zygotes moved around the drop with the denudation pipette. At 15 seconds we began to collect the oocytes (keeping them at the end of the pipette). Finally the zygotes were placed into the hole of the Rapid-i™ device, which was placed inside the polypropylene straw and sealed using the ultrasonic sealer (Vitrolife AB, Kungsbacka, Sweden).

(b) Warming

Vitrification solutions supplied by (Vitrolife, Gothenburg, Sweden) were used in the warming of zygotes. RapidWarm™ solutions are composed of four separate solutions: warm solution1, warm solution2, warm solution 3 and warm solution 4. Liquid nitrogen was collected and the vitrification box filled to the surface. The first straw was collected from the storage tank and in its cryocaine and transferred to the vitrification box. The straw was placed into one of the holding positions, here the top of the straw was cut just above the black end of the Rapid-I and the forceps used to lift the Rapid-I out of the straw (just enough to grasp with your fingertips). The Rapid-i™ was transferred immediately into Warm1 (ideally this should be done within 2 seconds, starting from the time the rapid-I is removed from the liquid nitrogen). The zygotes remained within Warming medium for 1 minute before zygotes were then moved into Warming medium 2 for 3 minutes, followed by Warming medium 3 for 5 minutes and finally into Warming medium 4 for 5 minutes. Finally they were transferred to an appropriate pre-prepared culture dish containing G1v5plus culture media (Vitrolife, Gothenburg, Sweden).

6.6.3.3 Vitrification of oocytes using an open based vitrification system

(a) Vitrification – cooling

Equilibration and vitrification solutions were dispensed into individual wells of four-well nunc dish and left to equilibrate RT for at least 30 minutes prior to use. Using a denudation pipette (vitrolife AB, kungsbacka, Sweden) oocytes/embryos were transferred to Equilibration medium 1 (1ml)(Origio™, Medicult, UK) here they remained for 5 minutes before they were transferred to the vitrification medium (1ml)(Origio™, Medicult, UK) for <1 minute. Using the Stripper-CC® micropipette oocytes/embryos were immediately loaded onto the tip of the McGill cryoleaf ™ vitrification device in a drop of less than 0.5µl (Origio™, Medicult media. Mellehaven 12, 4040, Denmark). The vitrification device (McGill cryoleaf ™) was then submerged directly in liquid nitrogen, then using a pair of large tweezers the lid of the device was also submerged in liquid nitrogen and placed over the tip of the device. These were then transferred to pre-labelled cryocaine filled with liquid nitrogen for indefinite storage in liquid nitrogen tank.

(b) Warming

Warming media (Origio™, Medicult media Mellehaven 12, 4040, Denmark) was allowed to equilibrate at 37^{o}C and all other media to RT for at least 30 minutes prior to use. Vitrification carrier was collected from liquid nitrogen (LN_2) storage container and transferred to a portable vessel containing LN_2 until transfer into warming media. Using tweezers vitrification device was held with area containing the embryo immersed in the liquid nitrogen whilst lid was removed, as quickly as possible, device was transferred to warming medium (2ml, 37^{o}C) here oocytes/embryos remained for 3 minutes. Using a denudation pipette (Vitrolife™ AB, Kungsbacka, Sweden) embryos were transferred and incubated in two subsequent dilution medium containing wells (2ml) for 3 minutes each, and a further two wash (2ml) solutions for 3 minutes each (Origio™, Medicult media. Mellehaven 12, 4040, Denmark). Oocytes/embryos were then transferred to appropriate equilibrated culture medium, where they were allowed time for post-warming recovery.

6.6.3.4 Vitrification of oocytes using an open based vitrification system

(a) Vitrification- cooling

Solutions supplied by Vitrolife must first be equilibrated to 37^{o}C prior to use (Vitrolife AB, Kungsbacka, Sweden). Samples are initially incubated in the first G-mops-like solution where they can remain for between 5-10 mins. Oocytes/embryos are then transferred to the second solution, where they remain for a further 2 minutes. Once samples have been transferred to the second solution the outer-sleeve, in which the Rapid-I™ is placed within during storage is plunged into the liquid nitrogen containing tank, with a metal rod which is supplied with each device. The metal rod remains within the straw. With 30 seconds remaining a 20µl droplet is made on the bottom of a falcon dish. Then with 10 seconds remaining the samples are picked up from the second solution using a denudation pipette and transferred to the a 20µl droplet, here they are then washes around the drop for 15-20 seconds with 10 seconds remaining oocytes/embryos are collected using the denudation pipette in a minimal volume of vitrification media and loaded onto the Rapid-i™ vitrification device.

(b) Warming

Oocytes were warmed at a later date (rehydrated using a serial dilution of sucrose). The straw was cut just above the black end of the Rapid-I and the forceps used to lift the Rapid-i™ out of the straw (just enough to grasp with your fingertips). Quickly the Rapid-I was transferred into Warm1 (ideally this should be done within 2 seconds, starting from the time the Rapid-i™ is removed from the liquid nitrogen). The oocytes remained within Warming medium 1 for 1 minute. Oocytes were then moved into warming medium 2 for 3 minutes, followed by Warming medium 3 for 5 minutes and finally into Warming medium 4 for 5 minutes. They were then transferred to an appropriate pre-prepared culture dish containing G-IVF culture media (Vitrolife™, Gothenburg, Sweden) for recovery.

6.6.4 Parthenogenetic activation

Here all activations were performed in 4-well Nunc dishes. Oocytes were initially incubated in G1 medium containing 1% HSA (700µl) (Vitrolife™, Kungsbacka, Sweden) supplemented with DMAP (7µl) (Sigma) and ionomycin (0.7µl) (Calbiochem) for 5 minutes, before they were transferred to the second well containing G1 medium containing 30%HSA(700µl) and DMAP (7µl) for a further 5 minutes, oocytes were then transferred to a third well containing G1 medium containing 10% HSA supplemented with DMAP (7µl) here they remained for 5 hours. Oocytes were finally transferred to the last well containing G1+HSA (700µl) and here they remained until they were checked the following morning for signs of PN development. Successfully activated oocytes were cultured in G1 medium containing 10% HSA until day 3 of development and were then transferred to G2 medium containing 10% HSA until day 7.

6.6.5 Imaging metaphase II (MII) spindle

Metaphase II oocytes were transferred to a glass bottom dish containing G1v5 Plus medium (Vitrolife, Gothenburg, Sweden). Oocytes were immobilised with a holding pipette and the spindle visualized using polarized light birefringence (Oosight Meta Imaging System, Cambridge Research and Instrumentation, CRi).

6.7 Results

6.7.1 Vitrification of abnormally fertilised human zygotes using an open vitrification system

In the first part of this study I examined the 'open vitrification' system (McGill cryoleaf™ Origio) (Fig6.3). This requires direct submergence of the vitrification device in liquid nitrogen before a protective sleeve, which pulls down over the sample provides some degree of protection with LN_2 during storage. Using this approach the cooling rate can reach up 23,000^{o}C/min, and a warming rate of up to 42,000^{o}C/min (Kuwayama, 2007). However the protective sleeve the device is fitted with is not heat sealed and therefore samples are still exposed to liquid nitrogen in a capacity which prevents this from being recognised as a 'closed vitrification' device.

The open vitrification system (Origio method) utilises a two-step vitrification protocol to achieve successful vitrification, including an initial equilibration and a second vitrification solution. The equilibration solution contains the cryoprotectants at a significantly lower concentration (7.5% ethylene glycol and 1, 2-propanediol) allowing zygotes to be exposed to this solution for an extended period of time (5-15minutes). Before they are then exposed to the actual vitrification solution which contains the cryoprotectants at a higher working concentration (15% ethylene glycol and 1, 2-propanediol). Exposure to the vitrification solution should not exceed 1 minute due to the presence of the cryoprotectants at a higher concentration.

I conducted a pilot study using the open vitrification system where I vitrified a number (n=25) of abnormally fertilised zygotes (1PN and 3PN) for which I later assessed survival and development. Initial overall survival attainable was low at 48% (n=12/25). This was whilst following the protocol supplied with the vitrification solutions which stated embryos could be incubated for between 5-10 minutes within the first equilibration solution before transferring to the vitrification solution. Following these guidelines once initial shrinkage (indicative of dehydration) and re-expansion of the embryo to its original pre-vitrified state has been observed embryos could be transferred to the second vitrification solution. However the time taken for zygotes to shrink and re-expand often varied between zygotes and required continual monitoring.

On multiple occasions I observed rapid shrinkage and re-expansion of zygotes, which was often accompanied by ‘blebbing’ around the cytoplasmic membrane, and ultimately reduced survival. Resulting in a number of zygotes degenerating during the actual vitrification procedure (n=20) (Fig6.4).

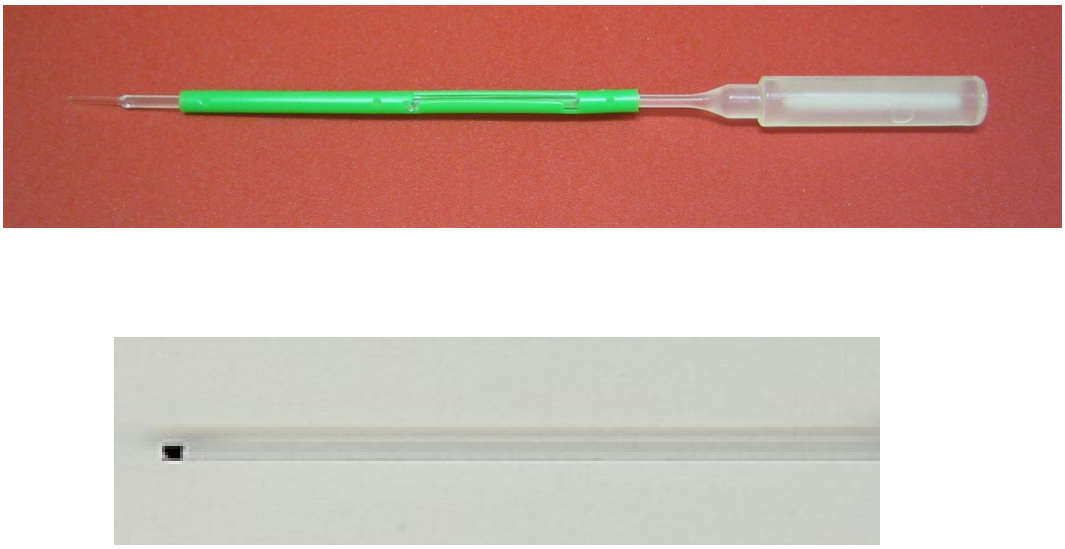


Fig6. 3 McGill cryoleaf® vitrification device

Above images represent the ‘open system’ vitrification device, which acts to increase the cooling rate via direct contact with the liquid nitrogen (McGill cryoleaf™, Origio Mellehaven 12, 4040, Denmark).

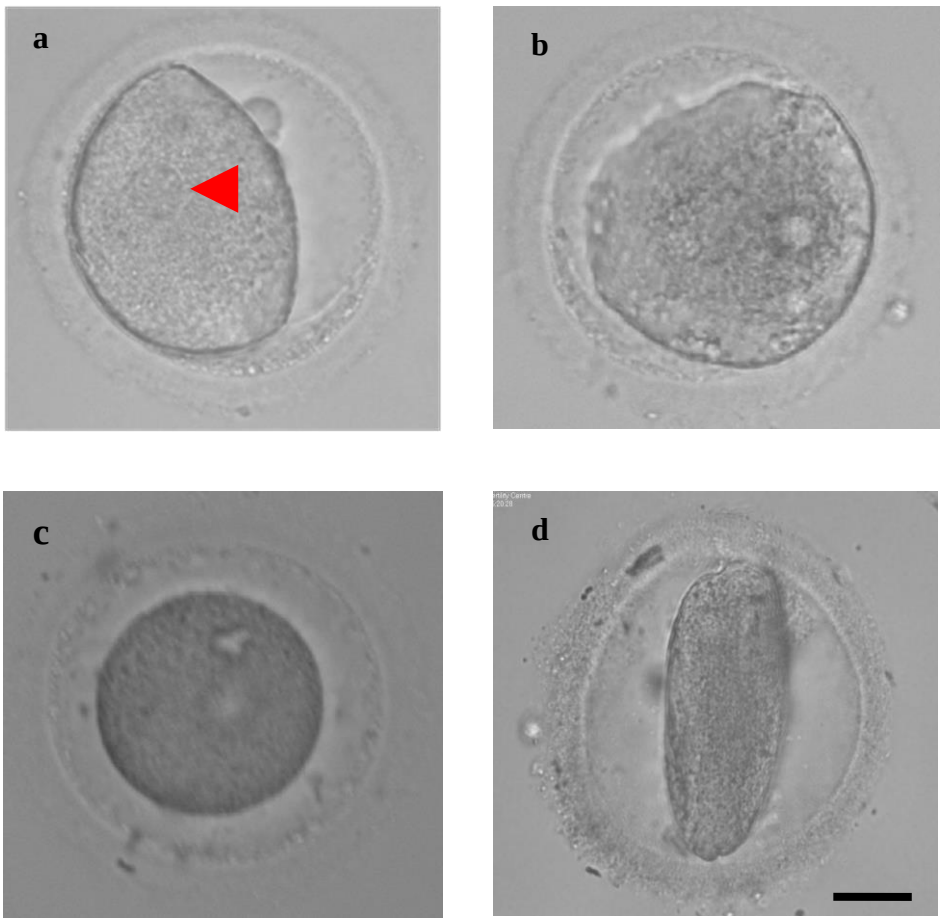


Fig6. 4 Observations of embryos following exposure to the 'open system' vitrification solutions

Several zygotes (a) failed to re-expand and (b) lysed and (d) degenerated following incubation in the initial equilibration solution. Scale bars, 50µm.

6.7.2 Optimization of the 'Open vitrification system' protocol

In light of the poor survival I took several steps to optimise the protocol supplied with the 'open vitrification system'. This included; increasing the volumes of both the equilibration and vitrification solutions used to vitrify with from 250µl to 500µl. The number of zygotes vitrified per set of solutions (500µl equilibration and vitrification solutions) was reduced to 4 zygotes. This was implemented due to concerns over potential for dilution of vitrification solutions when transferring the zygotes in culture media and also between the vitrification solutions. As this could potentially alter the composition of the media and in particular the active components (permeating and non-permeating cryoprotectants) key to achieving successful vitrification could be altered rendering them useless.

The exposure time to the equilibration solution was extended to a maximum of 15 minutes. If zygotes had not fully re-expanded beyond which point a note was made and zygotes were vitrified regardless. However there is a consensus opinion that those zygotes which fail to undergo shrinkage and re-expansion during 15 minutes of exposure to the equilibration solution is indicative of a poor outcome and such that (Personal communication, Lyndon Miles) (Fig6.4 and Fig6.5).

Further attempts were made to minimize the volume of vitrification medium in which the zygotes were loaded onto the vitrification device prior to submerging. This was achieved by dragging away excess media by creating a small channel of excess media once the zygote had been placed onto the tip of the flange where zygotes are to be loaded. This was then removed using the same glass denudation pipette used to load the zygotes onto the vitrification device so as to leave only a thin film of vitrification media covering the zygote before the final plunge into liquid nitrogen. The volume of liquid nitrogen used for vitrification was increased from 0.5 litres to 1 litre. This ensured that devices were completely submerged following the plunge into liquid nitrogen and not compromised by vitrifying in the vapour phase.

6.7.3 Improvements in survival outcomes following modifications to the 'Open vitrification system' protocol

Following implementation of the revised protocol I attempted to vitrify a further n=72 abnormally fertilized human zygotes. This resulted in significant improvements in survival, where it was possible to attain obtain overall survival of 91.6% (n=66/72) for all those mono- and multi-pronucleate stage zygotes which survived the vitrification process combined. This was almost double the survival observed during initial experiments (48%) using the same device but before optimisation. Further analysis of the survival data gathered following implementation of the revised protocol, revealed that zygotes containing 1PN demonstrated reduced survival compared to those multi-pronucleate zygotes containing 3 PN (89.7%, n=35/39 vs 93.9%, n=31/33; P>0.05 (Fig6.6) (Table 6.1). The trend towards reduced survival observed for the mono-pronucleate (1PN) zygotes may be a result of physiological differences in properties in the membranes rendering the mono-pronucleate (1PN) zygotes more sensitive to the vitrification solutions.

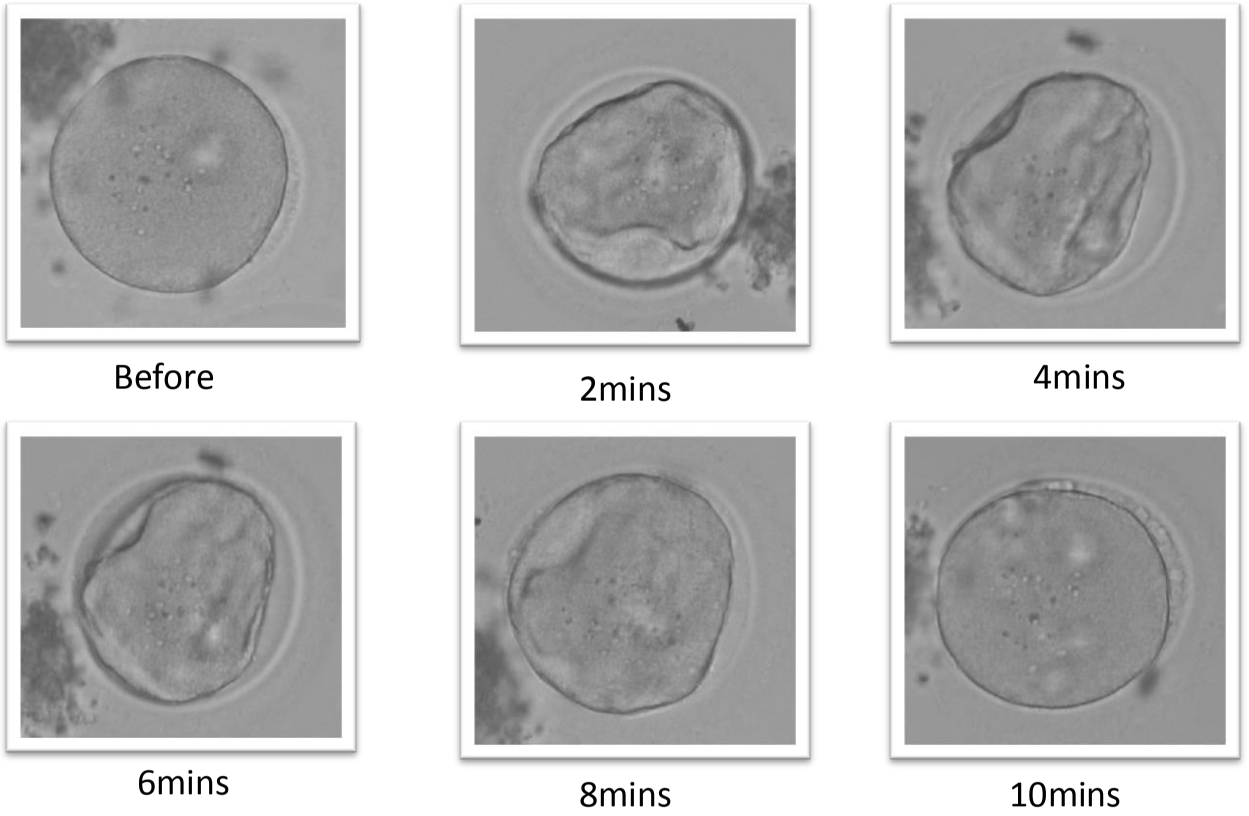


Fig6. 5 Vitrification cooling

Above images depicts a failed to fertilize (0PN) oocyte whilst incubated within the equilibration solutions supplied with the 'open' vitrification system. Here the oocyte gradually shrinks as cryoprotectants enter the cell. The cryoprotectants act to prevent ice-crystal formation during cooling-warming by forming hydrogen bonds with water molecules, within the cytoplasm of the zygote, therefore acting to de-hydrate the zygote. The equilibration solution contains cryoprotectants ethylene glycol and propane-2-diol at 7.5% and the actual vitrification solutions 15% ethylene glycol and propane-2-diol and also 0.5M sucrose). Scale bar, 25µm.

Survival Rate	Total Number survived	% Survival
1PN	35/39	89.7
3PN	31/33	93.9
Overall	66/72	91.6

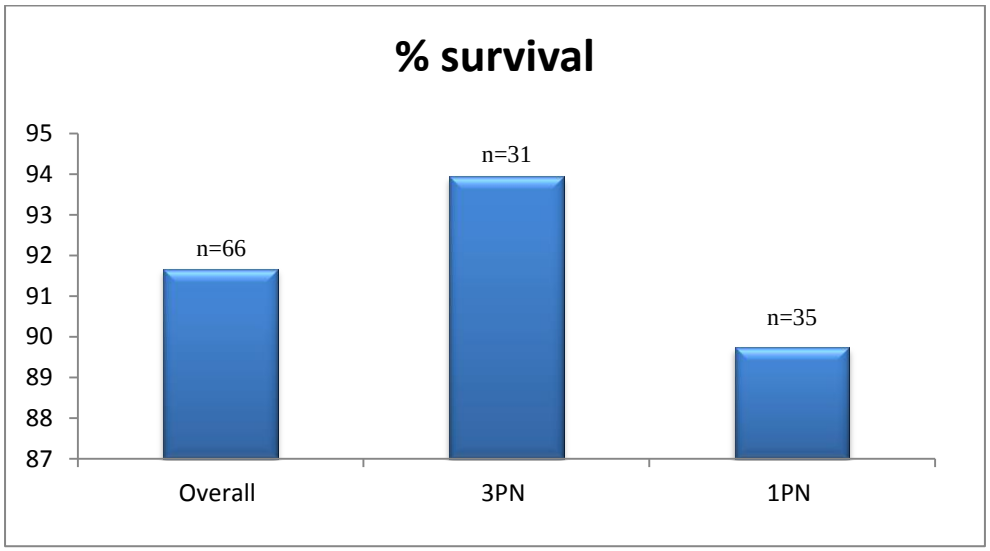


Fig6. 6 and Table 6.1 Overall survival observed for abnormally fertilised human zygotes vitrified using 'open vitrification system'.

Above figure represents survival outcomes determined for those abnormally fertilized human zygotes vitrified using the 'open vitrification system'. Overall survival was calculated to determine the total number of zygotes which survived the vitrification procedure regardless of pronucleate (PN) number. Here overall survival attained using the closed system was 91.6% (n=66/72). Independent survival outcomes were also calculated for 1PN zygotes as, 89.7% (n=31/33) and 3PN zygotes as 93.9% (n=35/39).

6.7.4 Embryo development following vitrification using the 'open vitrification system'

Development was assessed for those zygotes which survived the vitrification procedure (n=32). I compared development data gathered for those zygotes which survived vitrification (n=32) to control development data gathered for non-vitrified (fresh) abnormally fertilised human zygotes (n=56). Here of those zygotes vitrified 25%, (n=8/32) attained development to the <4-cell stage, 44% (n=14/32) the 4-8cell stage and 31% (n=10/32) >8cell stage. In contrast data gathered on development for a fresh non-vitrified zygotes revealed that; 18%, (n=10/56) attained development to the <4-cell stage, 29% (n=16/56) the 4-8cell stage and 53% (n=30/56) >8cell stage. Development observed for those embryos developing from vitrified zygotes was therefore reduced compared to that observed for those embryos in the control group. However statistical analysis revealed that there was no statistical significant difference in the development attained by those vitrified and cultured embryos versus nom-vitrified and cultured embryos (*pvalue*<0.05) (Fig6.7 and Table6.2).

	<4-cell		4-8cell		>8-cell	
	Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)
Cultured fresh	10/56	8.9	16/36	28.6	30/56	53.6
Vitrified and cultured	8/32	23.0	14/32	43.8	10/32	31.3

Table6.2 Development attained by embryos vitrified using an 'open' vitrification system (Origio™) versus embryos non-vitrfied (fresh) cultured embryos

Above figure represents comparison of development observed for fresh cultured embryos (control) versus vitrified and cultured embryos, vitrified using the open vitrification system (vitrolife vitrification solutions and Rapid-i™). Overall development observed for zygotes vitrified using the open vitrification system was as follows; 25% (n=8/32), <4cell stage, 44% (n=14/32), 4-8 cell stage and 31% (n=10/32). This compared to development attained by fresh controls, with; 18% (n=10/56) <4cell stage, 29% (n=16/56), 4-8 cell stage and 53% (n=30/56). Statistical analysis revealed that the development attained by those vitrified zygotes was not statistically significantly different to that attained by non-vitrified and cultured zygotes.

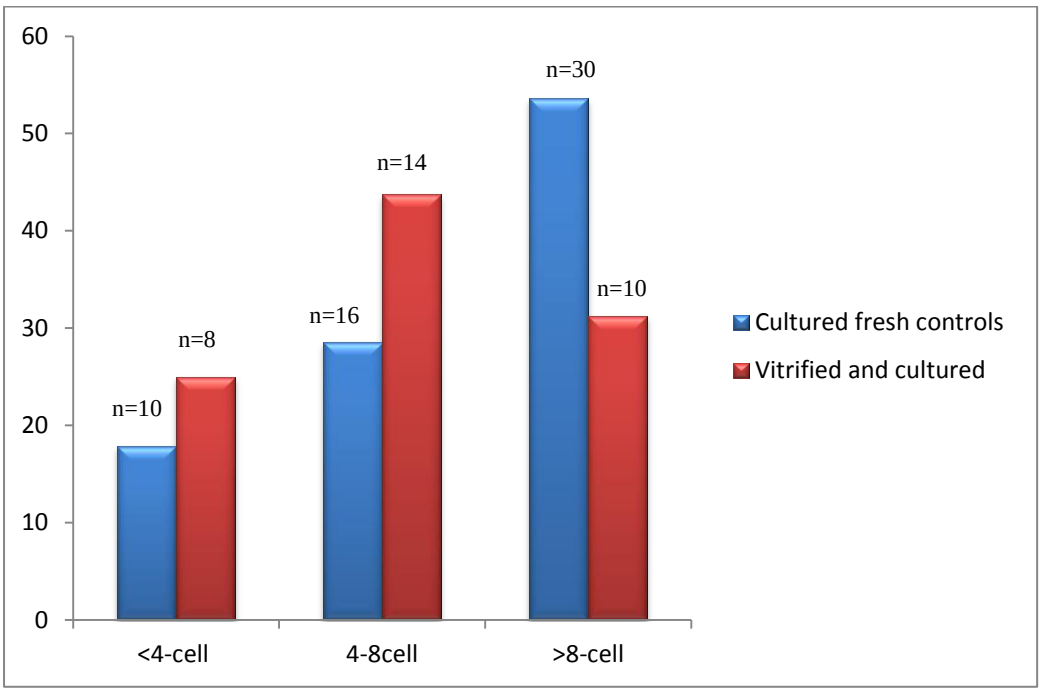


Fig6. 7 Embryo development following vitrification using an 'open vitrification system' (Origio™)

Comparison of developmental attained by embryos vitrified at the PN stage and cultured (n= 32) (red) using the closed vitrification system; versus non-vitrified (fresh) cultured control embryos. Overall development observed for embryos vitrified using the open vitrification system: 25% (n=8/32), <4cell stage, 44% (n=14/32), 4-8 cell stage and 31% (n=10/32) >8-cell stage. Development was also determined for those embryos treat as fresh controls, with; 18% (n=10/56) <4cell stage, 29% (n=16/56), 4-8 cell stage and 53% (n=30/56).

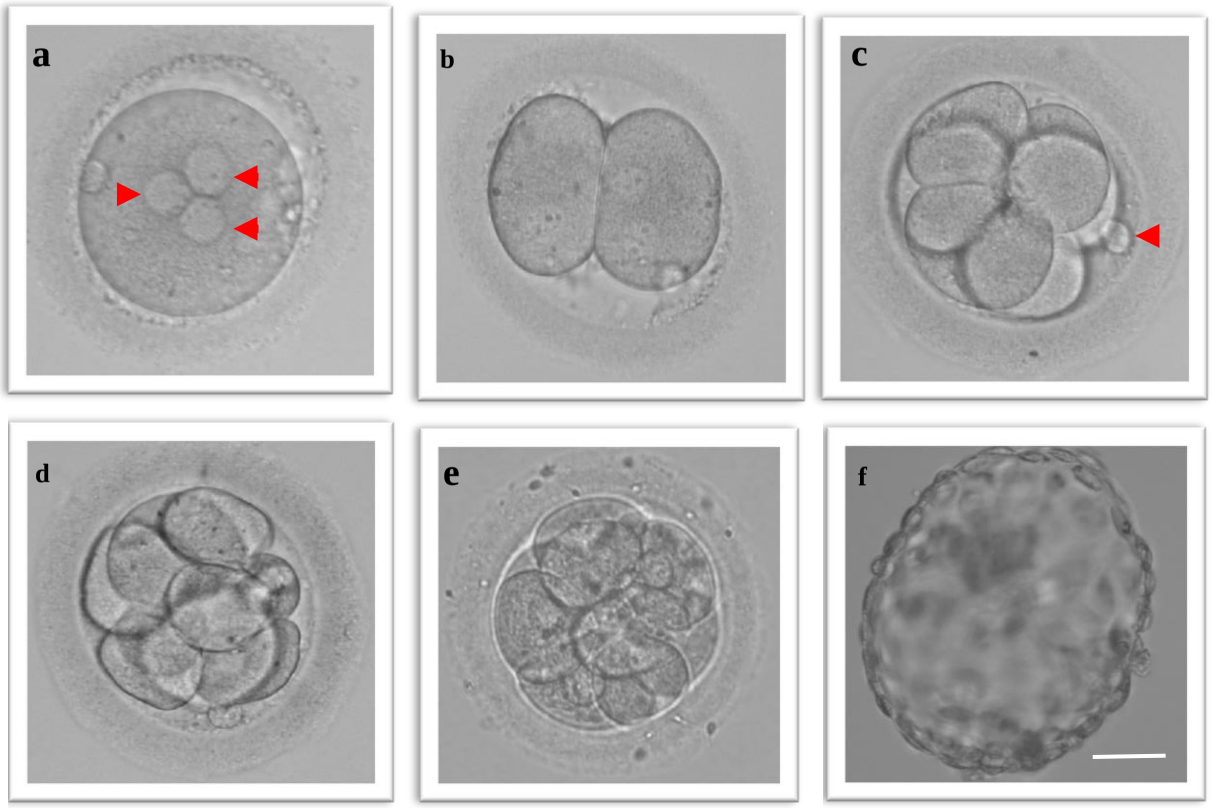


Fig6. 8 Development observed for vitrified and warmed abnormally fertilised zygotes

Above images represent abnormally fertilised embryos vitrified at the PN stage using the 'open' vitrification system and cultured following warming to assay developmental potential. Scale bars, 50µm.

6.7.5 Vitrification of abnormally fertilized human zygotes using a closed vitrification system.

The Rapid-i was developed as a vitrification device that is stored in a sealed straw under liquid nitrogen (Larman et al, 2011). It consists of a ploy(methyl methacrylate) rod and a polyvinyl chloride storage straw(Fig6.9). At the tip of the rapid-I is a flange that possesses a 50nL loading hole into which the oocytes/embryos are loaded (Larman et al, 2011). As a closed-system this has the advantage that it prevents direct contact with the liquid nitrogen in so avoiding the risk posed by open-systems of the potential risk of contamination by micro-organisms able to survive within liquid nitrogen However studies conducted using the Rapid-i™ in mouse oocytes have successfully demonstrated impressive survival rates and outcomes. Usually closed-systems are associated with a reduced cooling rate, which has previously been shown to negatively impact upon survival outcomes. However the cooling rate achieved with the Rapid-i™ which uses super-cooled air to vitrify has been determined as ~ 1,2000^{o}C/min. Indeed studies examining the device have been able to demonstrate that regardless of the fact that this is a fully sealed system, cell number is not detrimentally affected (equivalent to non-vitrified controls) as a consequence.

Fig6. 9 Rapid-i™ 'Closed' vitrification device

Above image represent the Vitrolife Rapid-i vitrification device. This is supplied in two parts consisting of a thin plastic strip containing a small hole and a plastic outer straw. Oocytes/embryos are loaded into the hole, (50nL) on the Rapid-I device using a denudation pipette in the minimal volume of vitrification solution and held in place I the hole by surface tension. (b) Vitrification box, designed as a holding vessel for the liquid nitrogen, supplied by vitrolife. The box is specially designed for vitrification with the Rapid-i vitrification device, due to the solenoid magnet built into the box to hold the Rapid-i outer straw in place whilst the device during the cooling and warming stages.

6.7.6 Validation of temperature for the 'open vitrification system'

Vitrification media supplied with the closed vitrification system (RapidVit oocyte™ and RapidWarm oocyte™ media supplied by Vitrolife™) is instructed to be used at 37˚C in order to maintain oocytes at physiological temperature during dehydration and re-hydration procedures. This therefore required all vitrification steps to be performed upon a heated stage in order to maintain solutions at the appropriate temperature throughout the process.

It was essential to ensure that the heated stage upon which the 4-well Nunc dish containing the vitrification solutions is placed during the vitrification process was maintained at 37^{o}C so as to ensure successful vitrification outcomes. Therefore before beginning to vitrify using the Vitrolife vitrification solutions we firstly validated the temperature of the heated stage. Here using a thermocouple (temperature monitoring device) the surface temperature was measured at varying places across the surface of the heated stage. The temperature did appear to fluctuate by 1-2^{o}C across the surface. Once the area on the heated surface, identified to maintain a steady temperature of 37^{o}C was found an area was marked out, to ensure the dish would be placed in the correct position during vitrification session. Air flow was also found to lower the temperature on the heated stage, therefore air-flow remained switched off whilst the vitrification procedure was performed.

Further steps were also needed to be taken to ensure that heat loss was minimised during the entire vitrification procedure. This included pre-aliqouting of the vitrification solutions. Vitrification solutions were supplied in vials of 10ml and dispensed into 1ml aliquots sterile capped tubes). Through creating the 1ml working aliquots of the vitrification solutions I was further able to minimize heat loss which would be encountered is dispensing solutions using a gilson pipette (personal communication L. Hyslop). A further advantage of creating the 1ml aliqouts was for improved sterility reasons.

6.7.7 Survival of zygotes vitrified using a 'closed vitrification system'

Overall survival was calculated for those abnormally fertilized human zygotes which survived vitrification using the 'closed system' (n=25). Here total overall survival was calculated as 96% (n=24/25) (Fig6.10 and Table 6.3). This was higher compared to that previously achieved using the open vitrification system 91.6% (Fig6.6 and Table6.2). However statistical analysis revealed that this was not statistically significant (*p-value*= 0.6733). Independent survival outcomes were also calculated to compensate for PN number. The total survival for 1PN zygotes was calculated as 93% (n=13/14) and for 3PN zygotes 100% (n=11/11) survival was possible. However only a small number of zygotes (n=25) were able to be vitrified using the 'closed vitrification system' due to the inconsistent nature of sample collection. Therefore it is difficult to infer if PN number affected survival outcomes.

6.7.8 Development following vitrification using a 'closed vitrification system'

Potential for onward development was also assessed for those zygotes which went onto cleave successfully following warming (n=16). Development obtained by those zygotes vitrified and warmed using the 'closed vitrification system' was compared to control data obtained for non-vitrified cultured zygotes. Of those vitrified zygotes which survived and demonstrated developmental potential (n=16) a total of 18% (n=3/16) attained development to the <4-cell stage, 13% (n=2/16) to the 4-8cell stage and 69% (n=11/16) >8cell stage. This contrasted to the control developmental data gathered for non-vitrified zygotes (n=56), with 18% (n=10/56) attaining development to the <4-cell stage, 29% (n=16/56) the 4-8cell stage and 53% (n=30/56) >8cell stage (Fig6.12 and 6.12) (Table6.4). Statistical analysis revealed that there was no statistical significant difference in the development attained by those vitrified and cultured embryos versus nom-vitrified and cultured embryos (*p value*<0.05).

Survival Rate	Total Number survived	% Survival
1PN	13/14	93
3PN	11/11	100
Overall	24/25	96

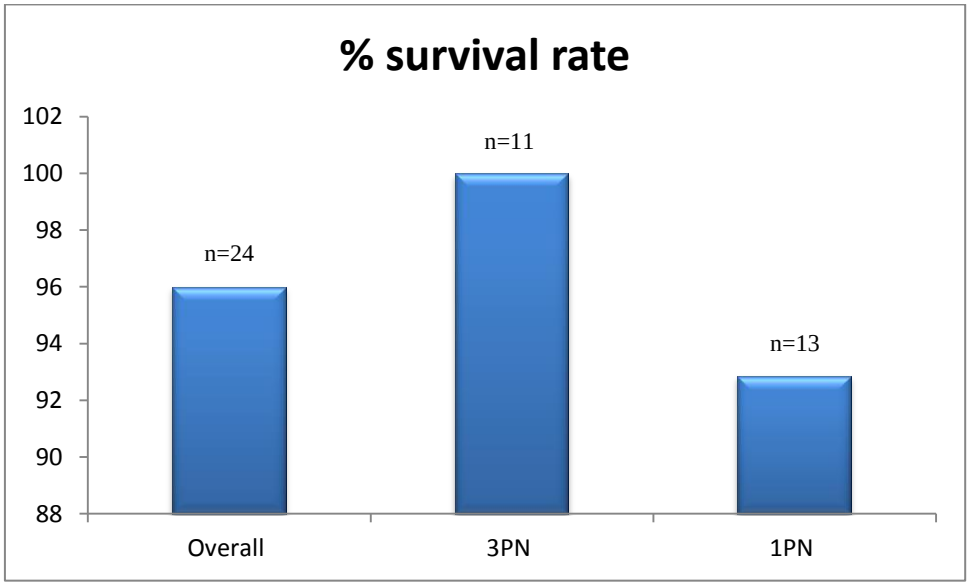


Fig6. 10 and Table 6.3 Overall survival observed for abnormally fertilised human zygotes vitrified using a 'closed vitrification device'

Above figure represents overall survival obtained for abnormally fertilized human zygotes vitrified using the closed vitrification system. Overall survival was calculated to determine survival regardless of pronucleate (PN) number and determined as 96% (n=24/25). Development data was further analysed to compensate for PN number and its effect on survival. The overall survival calculated for 1PN zygotes was 93%, (n=13/14) and for 3PN zygotes, 100% (n=11/11).

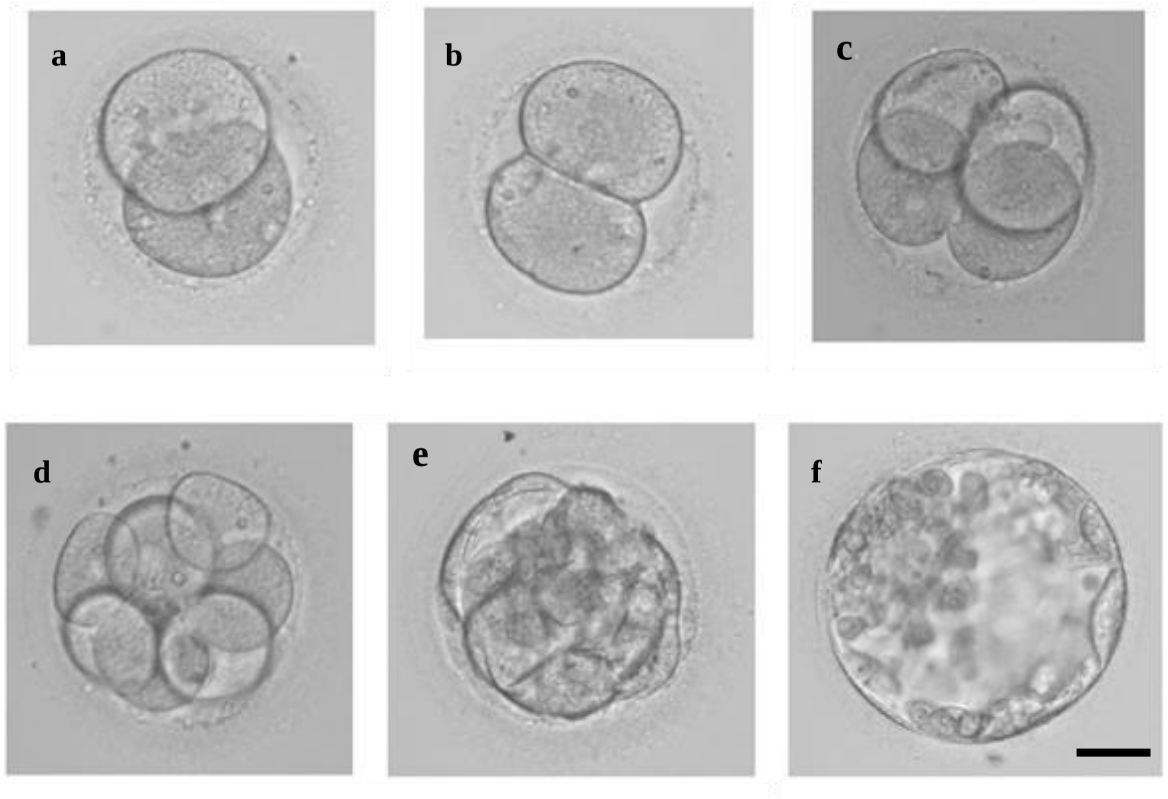


Fig6. 11 and Table 5.5 Development of embryos following vitrification, using a closed vitrification system (Vitrolife™ vitrification solutions and Rapid-i™ vitrification device).

Development observed for embryos vitrified at the single-cell zygote stage and then warmed cultured to ascertain development potential following vitrification using the ‘closed’ vitrification system . Scale bars, 25µm.

	<4-cell		4-8cell		>8-cell	
	Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)
Cultured fresh	10/56	18	16/56	29	30/56	53
Vitrified and cultured	3/16	19	2/16	13	11/16	69

Table 6.4 Comparison of development achieved for vitrified and cultured versus non-vitrified controls (abnormally fertilised human zygotes) using the closed vitrification device (Vitrolife™ vitrification solutions and Rapid-i™).

Above figure represents comparison of development observed for fresh cultured embryos (control) (blue) versus vitrified and cultured embryos (red) using the closed vitrification system (vitrolife vitrification solutions and Rapid-i™). Overall development observed for zygotes vitrified using the closed vitrification system was as follows; 18% (n=3/16), <4cell stage, 13% (n=2/16), 4-8 cell stage and 68% (n=11/16). This compared to development attained by fresh controls, with; 18% (n=10/56) <4cell stage, 29% (n=16/56), 4-8 cell stage and 53% (n=30/56). Statistical analysis revealed that the development attained by those vitrified zygotes was not statistically significantly different to that attained by non-vitrified and cultured zygotes.

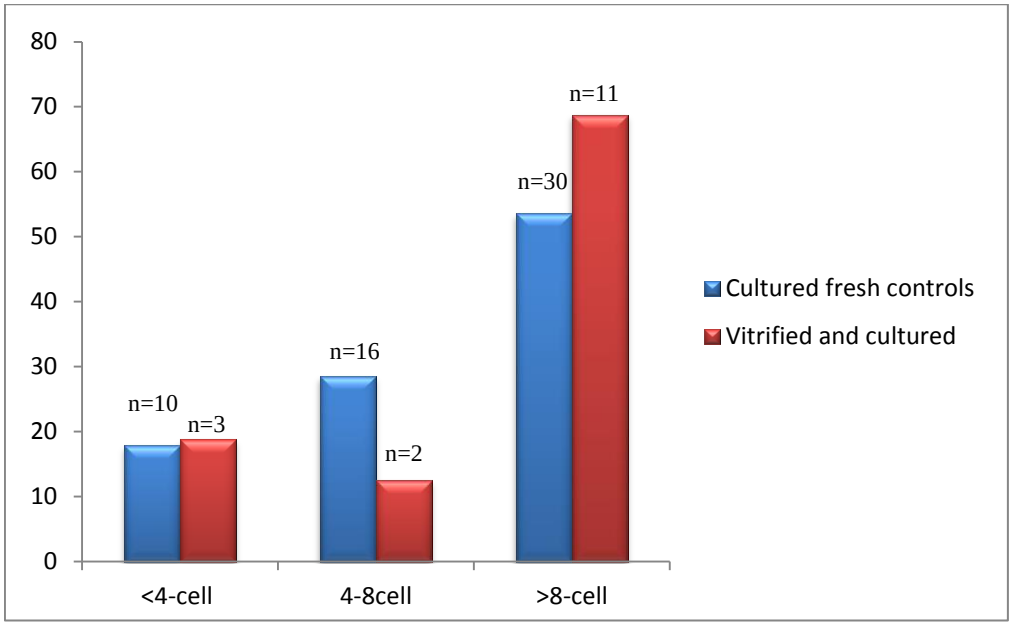


Fig6. 12 Comparison of development attained by embryos vitrified at the PN stage using the 'closed' vitrification system versus non-vitrified (fresh) controls.

Above figure represents comparison of development observed for fresh cultured embryos (control) (blue) versus vitrified and cultured embryos (red), vitrified at the PN stage using the closed vitrification system (vitrolife vitrification solutions and Rapid-i™). Overall development observed for zygotes vitrified using the closed vitrification system was as follows; 18% (n=3/16), <4cell stage, 13% (n=2/16), 4-8 cell stage and 68% (n=11/16). Development was also determined for those zygotes treat as fresh controls, with; 18% (n=10/56) <4cell stage, 29% (n=10/56), 4-8 cell stage and 53% (n=30/56) (*p value*>0.05).

6.8 Premature pronuclear (PN) breakdown observed following warming of abnormally fertilized zygotes vitrified using an 'open' versus 'closed' vitrification system

Following initial studies it soon became apparent that the time the pronuclei remained visible following warming and recovery (~2hours) varied considerably between the abnormally fertilised human zygotes vitrified. This we believed was influenced by the time they were able to be vitrified and which was subsequently dependent upon the time they were inseminated/fertilized the previous day and finally the time fertilisation checks were conducted and the zygotes able to be consented for use in the research study. From the initial study I conducted using the open vitrification system a significant number underwent pronuclear breakdown within a short time immediately following warming (n=22) or whilst in the recovery dish (n=15). This subsequently dictated the overall number of zygotes which could be considered to attempt manipulations with so as to determine if this was still possible to perform using zygotes which had been vitrified. Overall n=15 zygotes still contained visible pronuclei following warming and recovery for the open vitrification system. This compared to a total of n=14 zygotes using the closed vitrification system. Therefore despite the good overall survival able to be achieved for zygotes vitrified using both the open and closed vitrification systems, the potential to manipulate zygotes following warming and recovery is significantly compromised due to the limited time which pronuclei are visible for before they undergo PN breakdown. This is a factor which I did try to compensate for by making efforts to vitrify the zygotes as soon as from the time I received them however despite these efforts the overall number of zygotes which retained pronuclei was still relatively low. Given the risk that this might compromise the use of vitrified zygotes for the purpose of the pronuclear transfer technique, we did also explore the potential of vitrification at the MII stage (as discussed in chapter) to determine if this may provide an alternative route to bank oocytes from patients for future use when the technique has been established for clinical use (Fig6.12).

	Open vitrification system	Closed vitrification system
% of zygotes containing visible PN immediately following warming	69% (50/72)	87.5% (21/24)
% of zygotes containing visible PN following ~2hr recovery period	50% (15/30)	66.6% (14/21)

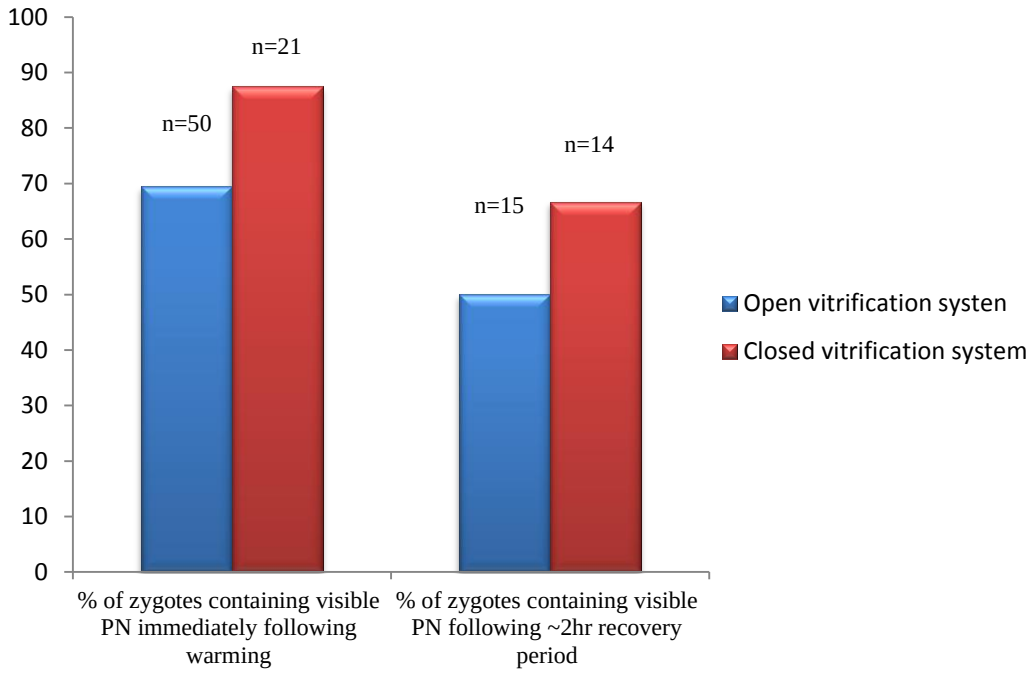


Fig6. 13 and Table 6.5 Comparison of PN breakdown following warming and recovery of abnormally fertilised human zygotes vitrified using the 'open' versus a 'closed' vitrification system

Above figure depicts the percentage of zygotes which retained visible pronuclei immediately following warming and those zygotes for which pronuclei could still be observed within after the 2hour minimum recovery period. This was analysed for zygotes vitrified using both the open vitrification system (blue) and closed vitrification systems (red).

6.9 Vitrification of oocytes

6.9.1 Vitrification of oocytes using a 'open' vitrification system

Initially a small number of oocytes (n=15) were vitrified using the open vitrification system. However the overall number of oocytes which survived upon warming was low, with a number of the oocytes noted to have suffered cryoinjuries, most likely as a result of sub-optimal vitrification. Here cryoinjuries are defined as visible morphological damage, which include the; vacuole-like structures within the cytoplasm, shrinkage of the cytoplasm away from the *zona pellucida* and granularistion and darkening of the cytoplasm (Fig6.14).

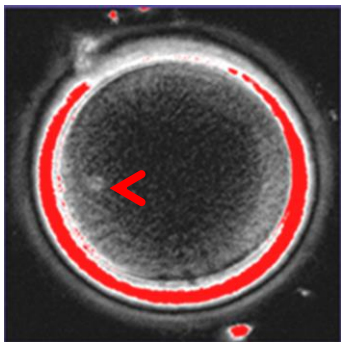
Previous studies have shown that these cryoinjuries result from either increased exposure time to the cryoprotectants during the cooling process. In an attempt to prevent cryoinjuries occurring and improve survival, several modifications were made to the vitrification protocol supplied with the open vitrification system. This included increasing the time the oocyte was incubated within the first equilibration solution. Upon warming we adjusted the time spent in each of the warming wash solutions, in the hope that by increasing the time spent in the wash solutions this may help to improve removal of the permeating cryoprotectants from the oocytes.

Following implementation of the modifications overall survival increased from 46% (n=7/15) to 60% (n=30/50).

Fig6. 14 Images of oocytes which did not survive the vitrification process when vitrified using the initial protocol supplied with the 'open' vitrification system.

Above images represent the various types of cryoinjuries which were encountered in the initial attempts undertaken to vitrify failed to fertilize oocytes. These included degeneration upon warming(a) vacuole-like structures within the cytoplasm of oocytes and embryos(b), cytoplasmic disturbances, most likely as a result of ice crystal formation within the cytoplasm (d) and(e) .

a



b

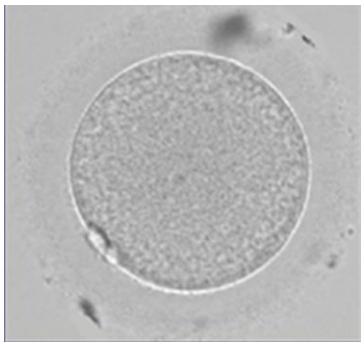


Fig 6. 15 Observing the spindle in vitrified and warmed MII stage oocytes

Above image confirms presence of intact spindle (indicated by red arrow) (a) imaged for a vitrified and warmed mature oocyte, using Oosight. (X40, magnification) (b) DIC image of the same vitrified oocyte.

	Fresh Oocytes (21)	Vitrified and Warmed Oocytes (30)
Overall survival	30	50
Overall survival	-	30/50 (60%)
spindle Present	12/21 (57%)	20/25 (80%)
Activation potential	8/12(66.6%)	12/20(60%)

Table6.8 Survival outcomes following vitrification using an open based vitrification system (Origio™ method)

The above table represents the current outcomes with regards to the vitrification of metaphase II stage oocytes. Here we vitrified a total of 30 oocytes, which we subsequently warmed at a later date. Upon warming these embryos were graded upon the morphological criteria we have previously described (Fig4.3). Oocytes vitrified using the open vitrification system demonstrated 60% survival. A select number of oocytes which survived (n=30) were then examined for the presence of a spindle using polarized light birefringence (Oosight Meta Imaging System, Cambridge Research and Instrumentation, CRi). A number (n=20) of those oocytes which were found to contain a spindle were the n considered for parthenogenetic activation (n=12/20). We also non-selectively allocated a number of oocytes to a control group, which were also checked using polarized light birefringence and once again a select number found to contain visible spindles parthogenetically activated.

6.9.2 Parthenogenetic activation

Those mature oocytes which survived the vitrification and warming process and later found to contain a spindle when imaged using polarized light birefringence (Oosight Meta Imaging System, Cambridge Research and Instrumentation, CRi).were then considered for parthenogenetic activation (n=20)(Fig6.15 and Fig6.16) and (Table 6.8). Parthenogenetic activation can be induced in mammalian oocytes by a diverse range of chemical compounds and physical conditions (Gook *et al*, 1995). A number of these chemicals contain hydroxyl (–OH) groups, and our commonly employed as cryoprotectants including propane-2-diol (PROH), which has previously been shown to be a potent parthenogenetic activator of mouse oocytes (Cuthbertson, 1983; Shaw and Trounson, 1989; Van der Elst *et al*, 1992; Gook *et al*, 1995). Several Metaphase II (MII) stage oocytes were determined to have undergone spontaneous activation following warming without intervention (n=8). However we did attempt to activate a number of oocytes (n=20) vitrified using the open vitrification system which were known to have retained their spindle. Indeed we were successfully able to activate a number of the vitrified oocytes, which we determined by the appearance of a pronuclei the following day (n=12/20(60%) (Table6.8). Unfortunately *in vitro* development observed following parthenogenetic activation was poor, although we cannot be sure if this was related to the vitrification process or the parthenogenetic activation and exposure to chemicals used for this purpose. However several demonstrated an attempt to cleave, and some even developing to morula stage (n=2). However due to the high degree of fragmentation and poor morphology of these embryos cell number was often difficult to judge.

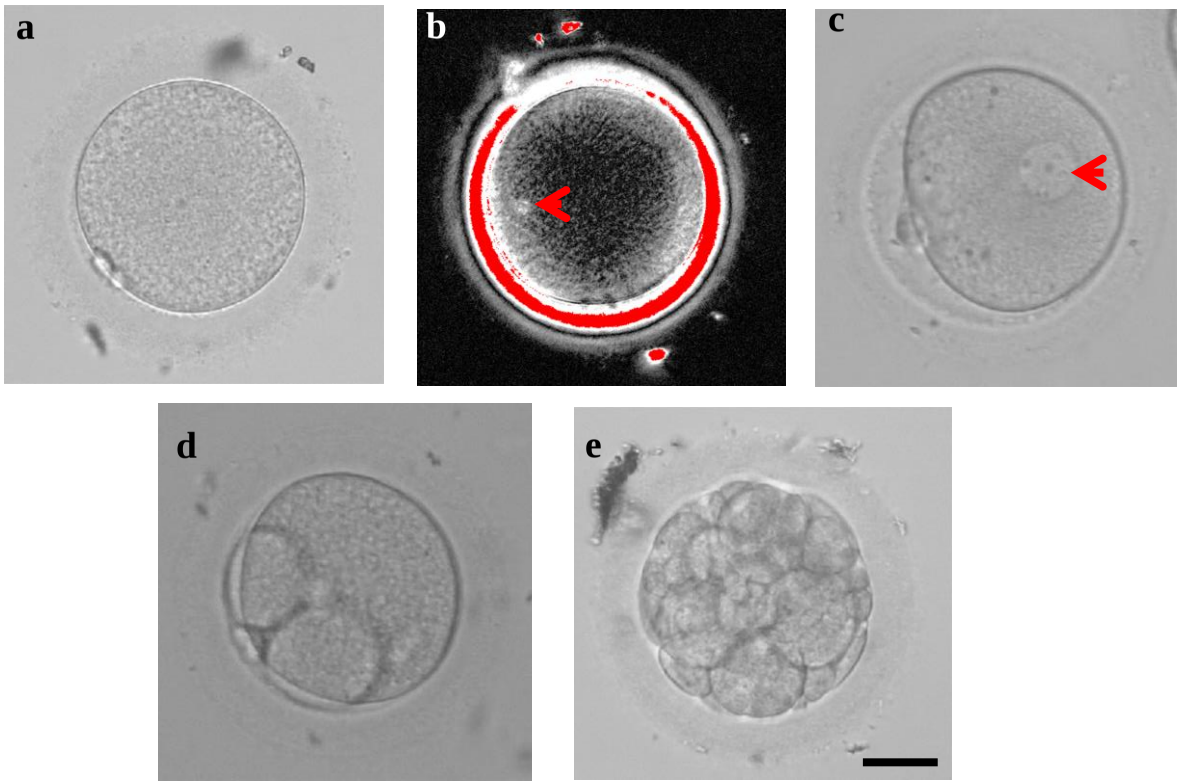


Fig6. 16 Parthenogenetic activation of a oocyte vitrified using a closed vitrification system

Those oocytes which were found to have an intact spindle when visualized using polar birefringence were considered for parthenogenetic activation. Above images represent an (a) bright field image of an oocyte following warming (b) which was subsequently imaged using oosight software to visualize the spindle (indicated by arrow). Following subsequent parthenogenetic activation the vitrified and warmed oocyte developed a pronucleus (PN) (c). However development was perturbed following parthenogenetic activation (d), as marked by the uncontrolled cell division and high degree of fragmentation (e). Scale bar , 50µm.

6.9.3 Vitrification of oocytes using a closed vitrification system

A closed vitrification system was also investigated for its potential to vitrify oocytes and determine if this could further improve on survival attained when vitrifying oocytes using an open vitrification system. Initially survival was low with a significant number of oocytes degenerating during the warming procedure. This was believed to be due to issues involving oocytes floating to the surface of the warming media attached to bubbles which were believed to be introduced during the loading of oocytes onto the vitrification device. This ultimately resulted in suboptimal warming conditions as oocytes were not fully exposed to the warming media and therefore degenerated as a result. Those oocytes which were attached to bubbles were unable to be visualized upon submersion of the vitrification device into the initial warming solution. In order to overcome this issue greater care was taken when loading the oocytes onto the vitrification devices in order to avoid the introduction of bubbles. Attention was also paid when warming oocytes and in particular transferring the vitrification device containing the oocyte into the first well containing the initial warming solution. Here the device was fully submerged in the well and not removed until the oocyte was visualized to have left the device, residing within the warming solution.

6.9.3.1 Oocyte survival using a closed vitrification system

Here we vitrified a number of oocytes (n=42) which included follicle reduction, *in vitro* matured (IVM) and failed to fertilize oocytes. These were then warmed (over a period of 3months) resulting in overall survival of 95.2% (n=40/42) (Table6.9). Oocytes were judged to have survived if they regained their pre-vitrified state, which included a fully intact *zona pellucida*, homogenous cytoplasm, lack of apparent incremental increase in inter-membrane space and no apparent cryoinjuries.

6.9.3.2 Examining for the presence of a spindle in vitrified oocytes using the imaging software Oosight™

Where possible oocytes were imaged using the Oosight™ imaging software (CRI,) to check for the presence of a spindle prior to vitrification however this was not always possible (if oocytes were vitrified within the clinical laboratory). A significant number

of those oocytes which were found to contain spindles prior to vitrification were also found to contain spindles when checked following warming, 87.5% (n=35/40)(Fig6.17).

6.9.4 Fertilisation potential of vitrified oocytes obtained from follicle reductions

A number of oocytes (n=13) obtained from follicle reductions were also warmed, and following adequate recovery time (~2hours) were imaged to check for the presence of a spindle using oosight (Research Instruments, Cornwall). Those oocytes which had good general morphology ad found to contain an intact spindle were then used to attempt fertilisation with (n=13). Here intracytoplasmic sperm injection (ICSI) was used to fertilize warmed follicle reduction oocytes, in favour of regular *in vitro* fertilization (IVF) due to the hardening of the *zona pellucida* regarded as an artefact of the cryopreservation process. (ICSI was performed by embryologists (L.Hyslop and S.Byerley). Fertilisation checks were performed the following morning (8am) to check for the presence of pronuclei (PN). A number of oocytes (n=8), which underwent ICSI, underwent normal fertilization as deemed by the presence of 2 pronuclei (n=6).

	Vitrified oocytes
Number oocytes vitrified	42
Total number of oocytes warmed	100% (42)
Survival rate following vitrification	95.2% (40/42)
Total number containing spindle after warming	87.5% (35/40)

Table 6.9 Vitrification outcomes for those oocytes vitrified and warmed using a closed vitrification system (Vitrolife™ oocyte vitrification specific solutions and Rapid-I™ vitrification device)

Here a number of oocytes were vitrified (n=42), these included failed to fertilise (0PN) and in vitro matured oocytes in order to determine the efficiency and feasibility of the closed vitrification system. Overall recovery following warming of vitrified oocytes was encouraging with 100% recovery achieved and overall survival of 95.2% (n=40/42). All oocytes which survived following warming were also examined for the presence of a spindle (n=40) >80% (87.5%, n=35/40) were found to contain a spindle when imaged using polarized light birefringence (Oosight Meta Imaging System, Cambridge Research and Instrumentation, CRi).

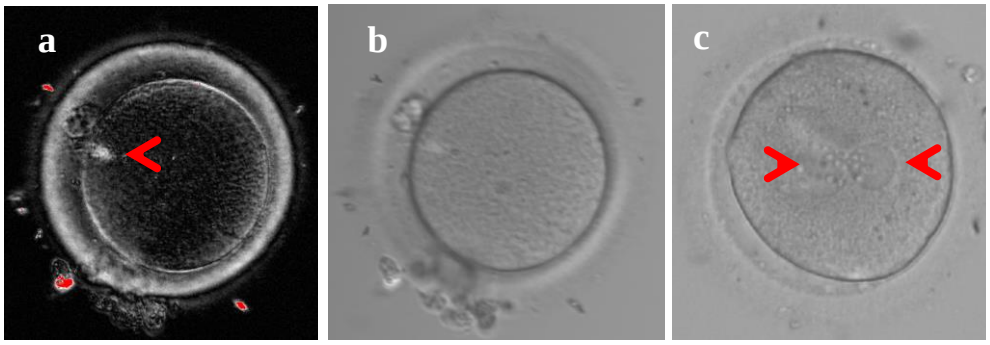


Fig6. 17 Images of vitrified and warmed Metaphase II (MII) stage oocytes using a closed based vitrification system (Vitrolife™ vitrification solutions and Rapid-I™ vitrification device)

Above images represent a follicle reduction oocyte which was vitrified using the closed vitrification system (Rapidvit oocyte and then warmed using Rapid-warm oocyte media, Vitrolife™). Following ~2hours recovery the warmed MII oocyte was examined using oocyte to check for the presence of a spindle (a) The MII oocyte was then fertilised using ICSI (vitrification causes hardening of the *zona pellucida*, making conventional IVF not possible) (c) The following morning fertilisation checks were made and two pronuclei

6.10 Discussion

The ultimate purpose of this study was to examine the efficiency and feasibility of vitrification at the metaphase II versus the pronucleate (PN) stage zygote.

In 2010 a paper was published which demonstrated the feasibility of the nuclear transfer technique pronuclear transfer (PNT) for preventing the transmission of mtDNA disease (Craven et al, 2010). All of the studies performed to date to examine the technique have used abnormally fertilized human zygotes, which are known to demonstrate a limited potential for onward development due to their abnormal chromosome complement (Personal communication L.Hyslop). Thus, in order to progress further with the research and better assess development and safety concerns regarding the PNT technique, studies must now be performed using normally fertilised zygotes.

Following the granting of the appropriate research licence by the HFEA it was made possible to create embryos, and use those which fertilize normally to assess the PNT technique. A recruitment drive is now underway to attract potential egg donors. However this has presented a logistical problem, for several reasons. As in order to perform PNT and assess mtDNA carryover to assess the success of the procedure, oocytes from two different egg donors would be required on the same day. This would therefore require to egg donors to undergo synchronous ovarian stimulation, so as to acquire oocytes from two different patients on the same day. However it is known that each individual's response to ovarian stimulation programmes differs considerably and therefore it is highly unlikely that both patients would be ready for egg collection on the same day. There is also likely to be a discrepancy in the number of oocytes retrieved from patients and therefore a high degree of wastage if we are not able to maximize their use. It is therefore imperative that a successful cryopreservation strategy is in place so as to be in a position to store either the pre-fertilized 'metaphase II' stage oocyte or following fertilization at the 'PN zygote' stage. I therefore set out to investigate the potential of vitrification at both the metaphase II oocyte and pronuclear (PN) stage zygote, in order to determine which would provide the highest post-thaw survival outcome.

Initially I investigated vitrification at the PN zygote stage. The PN stage is a unique stage during embryonic development which marks the transition from oogenesis to

embryogenesis. Here upon successful fertilization of an MII stage oocyte, the maternal and paternal nuclear DNA become visible as round membrane bound strictures, referred to as pronuclei. The presence of DNA within 'membrane bound structures' at the PN stage had led to the suggestion that it could potentially prove to be the optimal embryonic stage to cryopreserve embryos clinically (Damario et al, 1999; Orief et al, 2005). Indeed several studies have now examined cryopreservation, at the pronucleate stage using both controlled rate freezing and vitrification with much reported success (Veeck et al, 1993; Damario et al, 1999; Park et al, 2000; Damario et al, 2000; Isachenko et al, 2005; Orief et al, 2005; Kuwayama, 2005, 2007). However the PN stage zygotes unfortunately lacks distinctive morphological features which allow for successful grading prior to vitrification. Attempts have been made in the past to try and help establish a set of criteria which could be used in the establishment of a grading scheme for the PN stage zygote. Criteria which have been suggested could be used include; the position of the pronuclei in relation to the second polar body , the size and number of nucleoli within the pronucleus (Garello et al, 1999; Wittemer et al, 2000; Scott et al, 2000; Balaban et al, 2001; Payne et al, 2007).

Although even using these suggested schemes (Zygote scoring or Z-scoring) to grade at the PN stage, the grades agreed are not always highly accurate and the process can prove extremely time consuming. It therefore proves an incredibly difficult task to select zygotes for cryopreservation, compared to the selection of embryos at other embryonic stages (for example the cleavage and blastocyst stage) where successful grading schemes have been established. This has therefore limited the establishment of PN stage vitrification programmes in clinical practice as a result (Ludwig et al, 2000; Khalili et al, 2008). As the majority of IVF laboratories prefer to only cryopreserve those embryos which have good morphology prior to vitrification and therefore are likely to best survive the cryopreservation procedure, there are also cost and storage implications too. However my main reason for exploring vitrification at the PN stage was for the purpose of the PNT project. The idea that oocytes could be obtained from egg donors, fertilized and then cryopreserved until they could be used optimally. Therefore given the reports of success of cryopreservation strategies at the PN stage, it seems logical to explore the potential to vitrify at this stage.

However the methods used in the reports published describing successful outcomes differ a great deal. As there are many different approaches which have been used to

cryopreserve at the PN stage, including; controlled rate freezing, ultra-rapid freezing and more recently vitrification (Veeck et al, 1993; Damario et al, 1999; Park et al, 2000; Damario et al, 2000; Isachenko et al, 2005; Orief et al, 2005; Kuwayama, 2005, 2007). Not only did the majority of these studies use different cryopreservation strategies, many also used in-house designed vitrification devices and solutions, it therefore was not possible to adopt the same approaches previously utilised. Furthermore due to the recent resurgence in vitrification within IVF laboratories, new commercially available devices and vitrification solutions are continually being launched onto the market each promising improved survival and developmental outcomes.

Indeed there are many different types of vitrification systems now available commercially, including 'open' and 'closed' devices. It was therefore important as part of this study to examine both 'open' and 'closed' vitrification systems so as to determine how this affected survival outcomes. There is much evidence in the literature to suggest that the use of open based vitrification devices results in improved survival due to the higher cooling rate achieved from the direct contact with liquid nitrogen the devices permit (Mukaida et al, 2003; Kuwayama et al, 2005). However there is a disadvantage associated with the use of 'open' vitrification devices as they introduce a potential risk of contamination by micro-organisms able to persist within the liquid nitrogen even at temperatures of -196^{o}C (Bielanski et al, 2000, 2003, 2009). Therefore 'closed' vitrification devices offer an increased degree of safety and prevent the potential for contamination possible with 'open' based systems. I therefore also investigated the potential of a closed based vitrification device known as the Rapid-I. This is a unique 'closed' vitrification device which makes use of super-cooled air in order to achieve survival outcomes similar in the order to those only once able to be achieved using 'open' vitrification devices.

Initially I examined the open vitrification system which makes use of the McGill cryoleaf (Origio™). This solutions supplied with this open system comprises of a two-step vitrification protocol, which includes an initial 'equilibration solution' (ES) and a second 'vitrification' (V) solution. Zygotes/embryos are initially exposed to an equilibration solution containing the active cryoprotectants at a reduced concentration (7.5% ethylene glycol and 1, 2-propanediol) before they are incubated in a second solution (vitrification solution) again containing both active cryoprotectants but at an increased concentration (15% ethylene glycol and 1, 2-propanediol).

Following initial attempts at vitrifying zygotes using the open vitrification system I found that a considerable number of zygotes failed to fully regain their previous pre-vitrified state within the maximum exposure time of 10 minutes to the ES. As a result these zygotes proceeded to degenerate upon warming or survived but with 'poor' morphology and limited potential for onward development. A significant number of embryos were also observed to have lysed and also exhibit membrane 'blebbing' upon warming. There is evidence within the literature which have reported observing similar 'membrane blebbing' following extended exposure to vitrification solutions containing ethylene glycol (EG) (5mins), however this usually only occurred at high concentrations (>4M) (Hotamisligil et al; 1996; Valojerdi and Salehnia, 2005; Pogorelov et al, 2007). It is possible therefore that the initial poor survival I observed for zygotes following warming was a result of prolonged exposure to the cryoprotectants. However it is also likely that operator error and lack of experience with the technique also contributed to the reduced survival I observed. Indeed vitrification is a highly skilled procedure and requires great skill and manual dexterity in order to ensure zygotes/embryos are not over-exposed to the vitrification solutions and also loaded onto the vitrification devices within time constraints and minimal volume of vitrification media in order to achieve high survival outcomes. However following further experience with the technique and also optimisation of the conditions for the zygote stage of embryonic development a significant improvement in survival was able to be accomplished. Here previously survival was <45% however following the implemented improvement survival rose to 91.6%.

I therefore looked to examine the potential of a relatively new vitrification device known as the 'Rapid-i™' (Vitrolife). This uses super cooled air to improve survival outcomes once only able to be achieved using 'open' vitrification devices (Gardner and Larman, 2011). In order to achieve this, the Rapid-i is inserted into the super-cooled air found within the straw for instantaneous vitrification before it is finally sealed at the open end of the straw (occasionally referred to as the post-sealing method). This device also makes use of another novel adaptation a, 50nL hole present within the loading tip of the device. This I found aided greatly in the loading of zygotes, allowing the zygotes to be loaded in the minimal volume of vitrification media and excess media drawn off. Indeed minimizing the volume of vitrification solution the embryo is exposed to during storage, which has been shown to be result in improved survival

outcomes (Vajta et al, 1998; Lane et al, 1999; Kuwayama et al, 2007; Larman and Gardner, 2010; Zhang et al, 2011). Here it was possible to achieve over 96% survival (n=24/25) with the closed based vitrification system, which was slightly higher than that previously achieved when vitrifying using the open vitrification system (91.6%, n=66/72). However a substantially greater number of zygotes (n=72) were vitrified as part of the study examining the potential of the open vitrification system in comparison to the number vitrified using the closed vitrification system (n=25). However the overall survival I was able to achieve when vitrifying zygotes using both an 'open' and 'closed' system was comparable to those previously published data (Park et al, 2000; Isachenko et al, 2004; Kuwayama et al, 2007)

It was also important to ascertain that developmental potential was not jeopardized by the vitrification process. Therefore potential for onward development was assessed for zygotes vitrified using both systems. Interestingly developmental potential of those zygotes vitrified using the 'open vitrification system' was lower than that able to be achieved by zygotes vitrified using the 'closed vitrification system'. Here the number of embryos which attained development to the >8cell stage when vitrified using the 'open vitrification system' was less than half of that able to be achieved using the 'closed vitrification system' (31% (n=10/32) versus 69% (n=11/16) respectively). Furthermore development obtained by those zygotes vitrified using the 'closed' vitrification system was comparative to the control development data with 69% (n=11/16) versus 53% (n=30/56) attaining development to the >8cell stage (Fig6.18) However it must be noted that there were differences in the number of embryos within each of the three groups ('open', 'closed' and non-vitrified), which may have slightly biased the data.

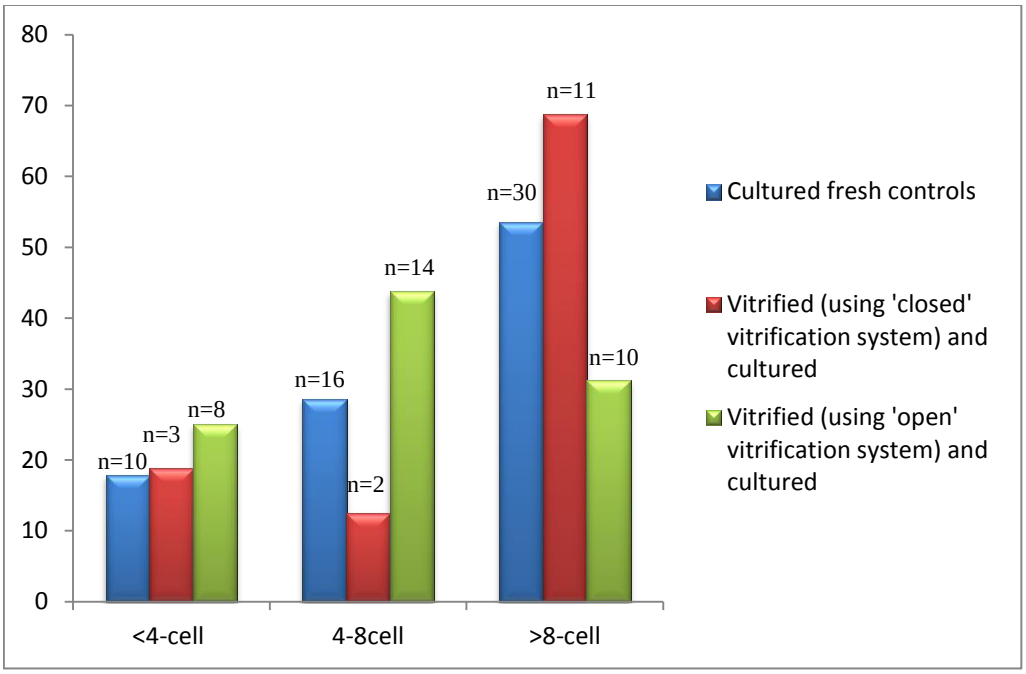


Fig6. 18 Comparison of embryonic development attained by embryos vitrified at the PN stage using an 'open' versus 'closed' vitrification system and in contrast to that observed for non-vitrified (fresh) cultured controls

Comparison of development attained by embryos vitrified at the PN stage using a 'closed' (n= 16) (red), versus 'open' (n=32) (green) vitrification system and how this compared to non-vitrified (fresh) cultured control embryos. Overall development observed for zygotes vitrified using the closed vitrification system was as follows; 18% (n=3/16), <4cell stage, 13% (n=2/16), 4-8 cell stage and 68% (n=11/16). Overall development observed for zygotes vitrified using the closed vitrification system was as follows; 25% (n=8/32), <4cell stage, 44% (n=14/32), 4-8 cell stage and 31% (n=10/32) >8-cell stage. Development was also determined for those embryos treat as fresh controls, with; 18% (n=10/56) <4cell stage, 29% (n=16/56), 4-8 cell stage and 53% (n=30/56).

However the lower survival observed with the open vitrification system was somewhat unexpected as open based systems are often praised for their high cooling rates, which promote high survival outcomes. However there are several explanations which may explain the difference observed in survival development attained by zygotes vitrified using the 'closed' versus 'open vitrification' systems. Firstly this may be a result of operator based errors. As the those zygotes vitrified using the 'open' vitrification system were the first zygotes I had attempted to vitrify, it is therefore likely that due to inexperience with the technique, survival and developmental potential was compromised as a result. These were said to be suitable to use at every stage of embryonic development from the oocyte to the blastocyst, their non-specific nature may have impacted on survival therefore. It is also possible that system were not optimal for the vitrification of abnormally fertilized human zygotes. Another possibility is the solutions used to vitrify with the open system. This also poses the question if the reduced survival was a result of the starting quality of the abnormally fertilized zygotes used.

As embryos obtained following abnormal fertilization may not have the same cryobiological properties and developmental potential as embryos obtained after normal fertilization (Noto et al, 1991; Joris et al, 1999; Park et al, 2000; Macas et al, 2011). However due to the scarcity and ethical issues associated with using normally fertilized human zygotes for validation and research purposes, the use of abnormally fertilized human zygotes as a model to evaluate different cryopreservation strategies is often the only option available. However multi-pronuclear zygotes are known to more closely resemble normally fertilized (2PN) zygotes and therefore often these demonstrate increased potential for development compared to their 1PN counterparts (Park et al, 2000; Isachenko et al, 2004)

Another interesting finding from this work revealed a difference in the overall survival observed for those 3PN zygotes (93.9%) versus 1PN zygotes (89.7%) vitrified using the 'open' vitrification system. Interestingly the same observation was also made for those zygotes vitrified using the 'closed' vitrification system (1PN (82%) and 3PN (90%)). Although these differences observed in survival between the 1PN and 3PN zygotes was not found to be statistically significant, given the low sample sizes used in this study, it must be considered when assessing the outcomes of this study. Indeed there are reports of similar observations regarding differences in survival and

developmental potential between mono and multi-pronucelate stage zygotes following cryopreservation (Park et al, 2000; Isachenko et al, 2004; Macas et al, 2011). It is possible that the slightly reduced survival observed for those 1PN zygotes is related to physiological differences which renders mono-pronucleate zygotes oocytes more similar in nature to oocytes rather than fertilized embryos (zygotes), potentially making them more sensitive to the vitrification process in comparison to their 3PN zygote counterparts. Therefore solutions employed to vitrify oocytes best suited vitrification of 1PN zygotes. However the media supplied with the 'open' vitrification system is described as a 'one media suits all' and can therefore be used from the oocyte right up to the blastocyst stage of embryonic development. It is possible that the non-specific nature of the vitrification medium used when vitrifying zygotes using the 'open' vitrification system may have been altered outcomes.

However despite the success I was able to achieve with regards to the successful vitrification of abnormally fertilized human zygotes, there were several issues which I had not previously accounted for, when assessing vitrification at this specific stage of embryonic development. This included the premature breakdown of pronuclei. This presents the biggest problem, as the reason for exploring vitrification at the PN stage was for in order to explore the potential of cryopreserving zygotes obtained from egg donors and mitochondrial patients. It is also important that a cryopreservation strategy is optimised and in place in order to allow for zygotes to be stored until a time to allow for optimal PNT transfers to be performed. This is in order to overcome issues surrounding synchronization and discrepancies in egg numbers.

I found that following warming of the vitrified zygotes and allowing for adequate recovery time prior to manipulating it became increasingly difficult to visualize the pronuclei. This was a major problem as the pronuclei in the majority of zygotes were breaking down before I had time to remove the pronuclei. In some instances it was possible but incredibly difficult to remove the pronuclei due to the faintness of their membranes. This therefore presented several problems and significantly reduced the overall number of vitrified zygotes I was able to use for PNT experiments with fresh zygotes.

There are several explanations which may explain why I observed the premature breakdown of pronuclei upon warming. The timing of the mitotic cycle can vary between fertilised oocytes, with the pronuclei becoming visible anywhere from 3 up to

20 hours after sperm entry into the oocyte and the pronuclear membrane breakdown occurring between 22 to 34 hours (Feenan and Herbert, 2006). It is reasonable to postulate therefore that this might account for some of the variability we observed in the expanse of time pronuclei were visible for in zygotes following warming. Another factor which may have contributed is the fact that this study made use of abnormally fertilised PN stage human zygotes which were only obtained once all fertilisation checks had been completed, and therefore the pronuclei in some zygotes were close to PN breakdown prior to vitrifying. However vitrification has previously been reported to impact on zygote morphology, with around 15% of zygotes failing to maintain pronuclear integrity after warming, which has been associated with poorer outcome). It is therefore possible that the vitrification procedure was also involved in the breakdown of pronuclei, due to chemicals found within the vitrification solutions (e.g. cryoprotectants). Cryoprotectants included within the vitrification solutions have previously been shown to contribute to parthenogenetic activation of oocytes upon warming (Gardner et al, 2007). It is therefore possible that the cryoprotectants may have induced PN breakdown biologically.

Due to issues encountered with premature PN breakdown upon warming I then set out to investigate the potential of vitrification of oocytes as an alternative to PN stage vitrification. Following this approach oocytes would be stripped of cumulous cells to asses maturity and then vitrified if mature. The MII stage oocytes could then be warmed at a later date at which time they would then be fertilized using ICSI (due to hardening of the zona pellucida, which prevents routine *In vitro* fertilization being used to inseminate). This would allow greater control over the time at which oocytes could be fertilized, the time and day the transfer could be performed and ultimately help overcome the issues regarding pronuclei breakdown encountered following zygote stage vitrification.

However until recently, the cryopreservation of oocytes has in the proven elusive. This is due to the unique physiology of the oocyte rendering them extremely sensitive to changes in their external environment in particular changes in temperature which can lead to osmotic shock. However recent advances in both vitrification solutions and devices has now made it possible to successfully cryopreserve oocytes and achieve good survival outcomes. Successful cryopreservation strategies can result in overall survival of >90% being attained in comparison to the 50-65% with conventional slow-

freezing (Chian et al, 2009). Here as with the PN stage vitrification experiments I examined two different commercially available systems; an open vitrification system and later a closed vitrification system.

Initial investigations using the open vitrification system (Origio™) and solutions again yielded poor survival outcomes. In the initial stages it was only possible to achieve overall survival 45%. Here the same solutions used to vitrify the PN stage zygotes supplied with the 'open' based vitrification system were used. These solutions were formulated to allow vitrification from the oocyte all the way through to the blastocyst stage embryo. Allowing clinics to invest in media which could be utilised for all stages of embryonic development and in so allow for the vitrification process to be streamlined within clinics and avoid issues surrounding confusion over different vitrification solutions only able to be used for specific stages of embryonic development. The only aspect required to be tailored to the stage of embryonic development for which the solutions are to be used is the timings. Here the time required within the equilibration solution (ES) differs between whether you are vitrifying oocytes, zygotes, cleavage stage embryos or blastocysts. But the time required within the final vitrification solution remaining unchanged despite the embryonic stage.

A similar phenomenon observed when using the same solutions to vitrify the PN stage zygotes was 'membrane blebbing' observed for several different oocytes whilst incubated within the first equilibration solution. This was despite this incubation period within the ES solution not exceeding the recommended time given within the manufacturer's protocol supplied with the solutions. However it is possible that the concentration of the cryoprotectants included within the ES solution was not appropriate for the oocyte. However having previously observed similar 'membrane blebbing' when vitrifying the PN zygotes using the same solutions it is more likely that this is an issue associated with the solutions and not the oocytes or zygotes. In attempts to overcome the issues associated with 'membrane blebbing' I looked to alter the time oocytes were incubated within the ES solutions, ensuring solutions were mixed thoroughly before aliqouting, the temperature of the media and surface was optimal at the procedure was being performed at.

There are other steps that can be taken to help improve survival outcomes. This includes observing oocyte shrinkage and re-expansion whilst exposure to the equilibration solution. It is recommended that oocytes should be completely shrunken after 3–6

from initial exposure to the equilibration solution. After this, oocyte shape should start to recover, with full recovery expected within 9–15 min. For human oocytes, full-size recovery seems to be extremely important for the success of the warming procedure: if they have still not re-expanded to at least >75% of their original size, survival prognosis is low (Personal communication, Lyndon Miles).

I was only able to achieve 60% survival when vitrifying oocytes using the 'open' vitrification system. This survival was good but still not optimal and would result in great oocyte wastage. Therefore it was essential to examine other approaches in ana attempt to achieve higher survival outcomes. I therefore examined the potential of a closed vitrification system, for oocyte vitrification **(**Vitrolife™ vitrification solutions and Rapid-I™ vitrification device). This provided the same benefits as described previously when using this system to vitrify at the PN stage. Indeed using the 'closed' vitrification system and oocyte specific vitrification solutions it was possible to achieve 92.5% overall survival for those oocytes vitrified using this system (Fig6.19).

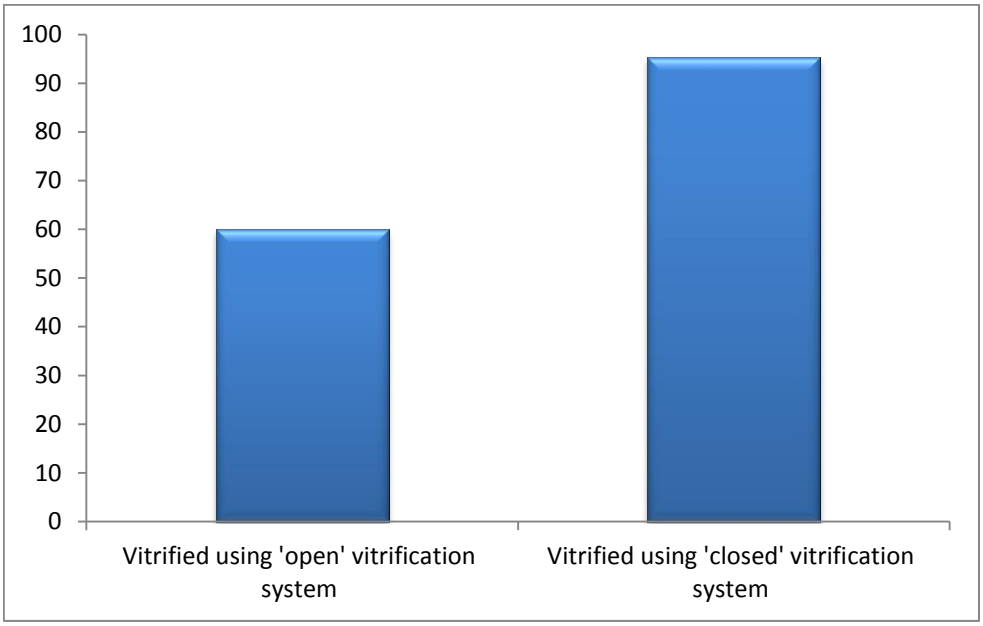


Fig6. 19Comparison of survival observed for oocytes vitrified using an ‘open’ versus ‘closed’ vitrification system

Above graph depicts the percentage survival obtained for oocytes vitrified using an ‘open’ (n=30/50) 60% versus ‘closed’ vitrification system (n=40/42) 95.2%.

There are several explanations as to why I was able to achieve higher overall survival when vitrifying using the 'closed' vitrification system. It is possible that this increase was attributable to the oocyte specific formulated equilibration and vitrification solutions supplied by the same manufacturer. This may therefore explain the lower survival outcomes achieved when vitrifying with the 'open' system as the vitrification solutions non-stage-specific. Allowing them to be used to vitrify; oocytes, zygotes, cleavage and blastocyst stage embryos. This is because each stage of embryonic development is known to possess different and unique physiology, therefore where one solution might best suit one stage of embryonic development it may not be best suited to all stages inclusively. It is therefore likely that the main reason for the difference in survival able to be achieved when using the different vitrification systems was influenced by the vitrification solutions.

Oocytes are known to exhibit differences in membrane permeability compared to embryos, which may affect the diffusion of cryoprotectants into the oocyte (Gardner et al, 2007). This is also a problem encountered with blastocyst stage vitrification and negatively affects survival outcomes (Vanderzwalmen et al, 2003; Kader et al, 2009). Oocytes are also known for their high water content which may also impact upon the time oocytes are required to be incubated within the ES solution in order to ensure that the cryoprotectants have been able to fully penetrate the oocyte and avoid the potential for ice crystal formation if not fully re-hydrated prior to vitrification. It is therefore incredibly likely that due to a combination of using oocyte specific vitrification solutions and performing the vitrification process at 37^{o}C improvements in overall survival were achieved.

It is possible that survival outcomes could be improved further in attempts to achieve 100% survival and minimize oocyte wastage by employing strict pre-freeze morphological criteria in order to assess oocyte quality prior to cryopreservation and ensure only good quality oocytes are selected. Screening criteria can include; zona pellucida thickness, size of perivitelline space, oocyte shape, cytoplasm colour and granularity, presence of vacuoles and first polar body morphology. It has also been said that those high-quality MII oocytes suitable for cryopreservation are colourless and of regular shape, with regular zona pellucida and small perivitelline space without debris, homogeneous cytoplasm and no vacuoles or granulations (De Sutter et al., 1996, Xia, 1997, Ebner et al., 2003, Parmegiani et al., 2008, Parmegiani et al., 2011).

At metaphase II, the maternal chromosomes are held suspended close to the cortex of the oocyte by a network of microtubules, called the meiotic spindle (Gardener et al, 2007). It is critical to ensure correct physical segregation of the chromosomes following polar body extrusion and the successful completion of meiosis in so ensuring the oocyte has the correct complement of genetic material (Gardner et al, 2007). It was therefore important to ascertain for those oocytes which survived with good morphology that they had also retained a spindle upon warming. Another reason it was important to ascertain that oocytes retained a visible spindle upon warming was due to concerns that the cryoprotectants included in the vitrification solutions, predominately the alcohols (ETOH and PrOH), are able to induce microtubule depolymerization (Vincent et al, 1990; Gook et al, 1993; Zenzes et al, 2001).

In order to examine the presence of a spindle within the oocytes following warmed I used polarized light birefringence (Oosight Meta Imaging System, Cambridge Research and Instrumentation, CRi) Polarized light microscopy uses liquid crystal optics to allow visualization and imaging of the meiotic spindle in living, unfixed MII stage oocytes, without compromising viability (Liu et al, 2000; Wang et al, 2001a; Rienzi et al, 2004; Gardner et al, 2007). This technique has therefore made it possible to visualize changes which can occur in the meiotic spindle following freezing and thawing, which may have been missed previously using conventional fixation and staining procedures. More importantly it allows for those oocytes for which a spindle has been visualized to be fertilized using ICSI and development assayed, which would not be possible if oocytes had to be fixed using PFA to examine the spindle.

Indeed upon warming I was able to observe a spindle in a high percentage of oocytes I vitrified using the 'open' (80%) and 'closed (87.5%) vitrification systems. I also found that in those oocytes I vitrified using the 'closed' vitrification system the spindle was visible almost immediately following warming and occasionally even appeared brighter than its pre-vitrified spindle. Indeed studies have shown that when human oocytes are vitrified involving an exposure to vitrification solutions at 37^{o}C, the meiotic spindle remains intact (Larman et al, 2006). It may therefore be the fact that combined effect of the super-cooled air used and the temperature (37^{o}C) the solutions are held while vitrifying and warming encourage spindle integrity and therefore further suggest are best suited to achieve successful oocyte vitrification.

A number of oocytes which survived following vitrification and warming with good observable morphology and a visible spindle were then considered for parthenogenetic activation. Parthogenesis is considered as the growth and development of embryos without fertilization by a male (Taupin, 2011). It occurs naturally in some species and can be induced artificially *in vitro* through the use of chemicals (Taupin, 2011). A significant number of those oocytes which I attempted to activate parthenogentically, went onto develop pronuclei. However development was often perturbed and 'blastic' in nature. The perturbed development I observed was most likely due to issues relating to current parthenogenetic activation protocols and the chemicals used to achieve this artificial activation. The imaging software used to image the spindle within the oocytes has a very low resolution, only allowing it to only be visualized as a flashing light within the oocyte. Therefore due to the low resolution it is possible that the spindle may have been scattered in some of the oocytes I attempted to activate using the chemical activation protocol, which would also explain the perturbed development I observed for some of these oocytes. Indeed similar studies who have examined *in vitro* development following parthenogenetic activation of oocytes also reported lower morphological grade embryo development following chemical activation, with a significantly lower proportion going onto cleave on day 3 (Paffoni et al, 2007). As fresh oocytes were not available to perform this study it is also worth noting that the oocytes I used in this study to examine oocyte vitrification were either in vitro matured (IVM) oocytes matured overnight or failed to fertilize oocytes, obtained three days following fertilization checks. This may have also affected outcomes in particular spindle integrity in failed to fertilize oocytes may have been compromised and likely prone to scattering by the time they were used.

At a later date we did also attempt to fertilize a number of oocytes which we had vitrified using the vitrification solutions, supplied by Vitrolife. This was only possible to now perform following receiving an HFEA license to perform ICSI for training purposes. Therefore those oocytes which were deemed to have good visible morphology, no observable cryoinjuries and visible spindle were used to attempt intracytoplasmic injection (ICSI). A number of these oocytes successfully went onto develop pronuclei, when fertilization checks were made the following morning. Almost all those embryos which successfully developed PNs went onto cleave, however development to the blastocyst stage was low. However when considering the development we observed with these embryos there are several factors we must allow

for. The rig that was used to perform the ICSI procedure was not equipped with Hoffman optics. The Hoffman Modulation Contrast system is designed to increase visibility and contrast in unstained and living material by detecting optical gradients (or slopes) and converting them into variations of light intensity (http://www.olympusmicro.com/primer/techniques/hoffman.html). This aids in the selection process of single sperm, to be used in the ICSI procedure, which is usually performed using morphology based criteria. Therefore the sperm selected for injection for those vitrified oocytes in this study was done so under sub-optimal conditions and may have impacted upon *in vitro* development observed. Furthermore the media used to hold the warmed oocytes and sperm preparation in during the ICSI procedure under normal circumstances would be G-MOPS (within the clinical lab) however this was substituted and G1v5plus used instead. G-MOPS is a HEPES buffered medium which allows for samples to be held at room temperature safely, G1v5plus however is not HEPES buffered and therefore requires to maintained in a modified environment which is held at $37^{o}C$ and supplied with $C0_2$. Therefore in substituting the media used to hold the samples during ICSI sperm quality may have been impaired and in future it may well be more appropriate to use G-IVF culture media instead of G1v5plus as this is specifically formulated for the handling of gametes. Although development was reduced in the vitrified and ICSI oocytes it was significantly better than that previously observed following parthenogenetic activation of oocytes.

Oocyte vitrification followed by ICSI has been shown is not inferior to fresh insemination procedures, with regards to both fertilization and embryo development rates and has been reported following several similar independent studies (Kuleshova et al, 1999; Yoon et al, 2003; Kuwayama et al, 2005; Rienzi et al, 2010). This study published examined embryo development in fresh versus vitrified mature oocytes following ICSI through a prospective randomized sibling-oocyte study. Here the fertilization rate obtained with fresh oocytes was determined as 83.3% (100/120) in comparison the fertilization rate (2PN) achieved with the vitrified and warmed group per sibling oocyte was determined as 76.6% (95/124). In all cases for both the fresh and warmed oocytes a total of three oocytes were made available for ICSI procedures. Fifty four patients (43.2%) were confirmed to have obtained a clinical pregnancy following a fresh cycle, of these 48 are on-going (beyond 12 weeks) (38.8%). Of the confirmed clinical pregnancies recorded in the vitrified and warmed group (37.5% per cycle and 38.5% per embryo transfer) 12 were currently on going at the time of publishing (as

given beyond 12 weeks of gestation) (30.0% per cycle and 30.8% per embryo transfer). This study utilized the cryotop vitrification device and media also supplied by the same company as the vitrification device Kitazato, which is marketed on the premise of its specific formulation for the vitrification of oocytes. The sample size of oocytes included in this study was much larger than our study however it does successfully demonstrate the strong potential of vitrified oocytes.

Chapter 7

Pronuclear Transfer (PNT) performed between vitrified and fresh abnormally fertilized human Zygotes

Chapter 7 Pronuclear Transfer (PNT) performed between vitrified and fresh abnormally fertilized human Zygotes

7.1 Introduction

After establishing it was possible to vitrify abnormally fertilized PN stage zygotes, the next aim was to determine if it was possible to manipulate using vitrified zygotes. It was also important to establish if mtDNA carryover was affected when using vitrified zygotes. Therefore for those zygotes which survived the manipulation process and showed onward development, mtDNA carryover analysis was performed.

7.1.1 MtDNA Heteroplasmy following Pronuclear Transfer performed using mouse embryos

Studies have examined the PNT technique using mice carrying a large-scale mtDNA deletion (mito-mice) (Nakada et al, 2001). The average level of mtDNA carryover was determined as 35% for the mice generated following nuclear transplantation (Sato et al., 2005). Here polar bodies were used as biopsy samples to determine carryover. However as we know now from more recent studies, polar bodies do not provide an accurate representation of the mutation load observed in the zygote, when sampled to analyse mtDNA mutation loads (Gigarel et al, 2011). It is therefore likely that this from of sampling did not provide a completely accurate account of the mtDNA carryover. Although the resultant progeny had an average of 11% mutated mtDNA in tail samples and did not develop any clinical symptoms associated with the mtDNA mutation. This therefore demonstrates that pronuclear transfer can be an effective method to prevent transmission of mtDNA disease.

Other studies have completed pronuclear transfer between embryos from different strains of mice and investigated the level of mtDNA carry-over in reconstructed embryos. One such study revealed that pronuclear transfer embryos analysed following manipulation contained on average 19% mtDNA of karyoplast origin, ranging from 16 to 23% (Meirelles and Smith, 1997). Most progeny derived from these embryos were found to contain less mtDNA from the pronuclear karyoplast but with some variation in heteroplasmy levels between tissues, ranging from 6% (lung) to 69% (heart).

Another study used the same approach and determined the level of karyoplast mtDNA in reconstructed embryos cultured to the blastocyst stage following pronuclear transfer (Brown et al., 2006). This study reported a similar level of 16.3±8.4% mtDNA of karyoplast origin and confirmed that it is possible to generate mice with low levels of mtDNA carry-over. Variation in heteroplasmy between tissues was also revealed, with levels ranging from 10% (muscle) to 37% (liver).

7.1.2 MtDNA Heteroplasmy following Pronuclear Transfer between human embryos

Having established that pronuclear transfer was compatible with onward development of human embryos, carry-over of donor mtDNA genotype was examined in reconstituted human pronuclear-transfer embryos (Craven et al 2010). Last hot cycle PCR restriction-fragment length polymorphism (RFLP) assays were developed for identified mtDNA variants and used to analyze mtDNA extracted from whole embryos. We found that there was variation in the amount of mtDNA genotype from the donor zygote transferred to the embryos receiving two transferred pronuclei (8.1% ± 7.6 (mean ± s. d.), $n = 8$).

Analyses were also performed to determine whether the proportion of donor mtDNA genotype also varied between blastomeres in the reconstituted embryos after transfer of two pronuclei. In one out of eight embryos no donor mtDNA was detected in any blastomere. In the other seven embryos analyzed, there was variation in the level of donor mtDNA genotype between blastomeres. Although the variation observed was similar to previous reports on heteroplasmic human embryos (Steffan et al, 2006; Tajima et al, 2007). However attempts made to further minimize the carry-over of donor zygote mtDNA, involving techniques to reduce the amount of cytoplasm contained within the pronuclear karyoplast. Analysis of donor mtDNA genotype carryover following improvements to the technique demonstrated that the mtDNA carry-over was significantly lower ($P < 0.005$), with four out of nine embryos now found to contain undetectable levels of mtDNA carry-over. The average mtDNA carry-over in all remaining embryos was less than 2% (1.68 ± 1.81% (mean ± s.d.), $n = 9$). These same embryos also revealed much less variation in mtDNA carry-over between individual blastomeres.

7.1.3 Mitochondrial distribution in cryopreserved human embryos

Studies examining mitochondrial distribution in human abnormally fertilized pronuclear stage zygotes and cleaved embryos frozen using ultra-rapid freezing have demonstrated its similarity to that observed for controls (Noto et al, 1993; Nottola et al, 2007). However other studies also examining mitochondrial distribution in vitrified abnormally fertilized human zygotes, reported observing the formation of large SER vesicles associated with mitochondria following ultra-structural analysis performed using electron microscopy (EM). These were said likely to be a result of the cryopreservation process and possible caused as a result of defects or damage to Ca^{2+} -regulation (Sathananthan and Trounson, 1989; Sathananthan et al, 1988b; Liu et al, 2001; Isachenko et al, 2003). There is also evidence that mitochondria ultrastructure and viability (as assessed by analysis of mitochondrial polarity) may be adversely affected by the cryopreservation procedure (Jones et al, 2004).

7.1.4 Manipulation of cryopreserved oocytes and embryos

There is a single report of a study performed to evaluate the feasibility of using cryopreserved oocytes for maternal spindle transfer (MST), using the nonhuman primate model. They used a commercially available vitrification kit (CRYOTOP) and determined high post-thaw survival and recovery of rhesus macaque MII oocytes. Furthermore following ISCI, 72% formed pronuclei, but only 6% developed to blastocysts. Thus, this cryopreservation method was suggested compromised blastocyst development, because blastocyst formation of fresh oocytes from the same cohort was 52%.

The same study also examined the ability to perform reciprocal MST between fresh and frozen-thawed monkey oocytes and examined fertilization and embryo development. When fresh spindles were transplanted into vitrified cytoplasts, fertilization after ICSI was impaired (50%) compared to controls (91%). Furthermore all embryos in this ST group arrested before reaching blastocysts, whereas 57% controls progressed to blastocysts. These MST results were similar to those seen with frozen-thawed intact controls. However, when spindles from vitrified oocytes were transferred into fresh

cytoplasts, fertilization (88%) and blastocyst formation (68%) rates were similar to fresh controls. These results suggested that vitrification causes damage primarily within the cytoplasm rather than to the spindle apparatus.

7.2 Materials and Methods

7.2.1 Pronuclear karyoplast removal

Zygotes were incubated in G1v5 Plus medium (Vitrolife) containing cytochalasin B (5µg/ml) and nocodazole (10µg/ml) at 37ºC with 7% CO2 for 10 or 30 minutes prior to manipulation and during the procedure. Manipulations were performed using an inverted microscope (Nikon Eclipse TE2000-U) equipped with a micromanipulation system (Integra Ti, Research Instruments). Zygotes were immobilised with a holding pipette and a small ablation made in the *zona pellucida* using a microsurgical laser (Saturn Active, Research Instruments). A customised biopsy pipette with an inner diameter (ID) of 28µm was inserted under the *zona pellucida*. The pronucleus and surrounding cytoplasm were then aspirated into the biopsy pipette as a membrane bound karyoplast. For transfer of a single pronucleus, a pronuclear karyoplast was removed from either a monopronucleate or tripronucleate donor zygote and transferred to a recipient zygote containing only one pronucleus. The recipient zygote was either a pronucleate zygote, which required no manipulation prior to transfer, or a tripronucleate zygote from which two pronuclei had been removed. Thus, the reconstituted zygotes contained two pronuclei. In experiments involving transfer of two pronuclei, pronuclei were removed either as two individual pronuclear karyoplasts or a single karyoplast containing both pronuclei. These karyoplasts were then transferred to an enucleated recipient zygote such that the reconstructed zygote contained two pronuclei.

7.2.2 Pronuclear karyoplast fusion

Pronuclear karyoplasts were transferred within a biopsy pipette to a 1µl drop of HVJE (GenomONETM-CFEX HVJ Envelope Cell Fusion Kit, Cosmo Bio Co) and a small volume of the suspension approximately equal to the volume of the karyoplast aspirated into the pipette. The pipette was then moved to a drop containing a recipient zygote.

The pipette was inserted into the zygote through a small ablation in the *zona pellucida* and the HVJ-E and pronuclear karyoplast aspirated into the perivitelline space, ensuring good contact between the karyoplast and plasma membrane. Fusion of the pronuclear karyoplast with the recipient zygote was confirmed visually and usually occurred within 10 minutes up to 1 hour after transfer. Manipulated zygotes were transferred to G1v5 Plus medium (Vitrolife) and cultured at 37ºC with 7% CO2. Embryos were transferred to G2v5 Plus medium (Vitrolife) on day 3 of development and cultured at 37ºC with 7% CO2 up to day 7. Embryos for mitochondrial DNA analysis were then transferred to sterile 0.5ml microfuge tubes and stored at -20ºC until DNA extraction.

7.2.3 MtDNA Sequencing

7.2.3.1 PCR amplification of the non-coding control region

The non-coding control region of the mitochondrial genome was amplified using two rounds of PCR amplification as described previously (Taylor et al., 2001) with the following modification: secondary PCR reactions were performed with 4 sets of overlapping M13-tailed primers (primer nucleotide positions: D1F: 15758-15777 and D1R: 019-001, D2F: 16223-16244 and D2R: 129-110, D3F: 16548-16569 and D3R:389-370, D4F: 323-343 and D4R: 771-752) with an annealing temperature of 58ºC.

7.2.3.2 Purification of PCR products for sequencing

PCR products were purified using ExoSap-IT (GE Healthcare) according to the manufacturer's instructions. 5µl PCR product was transferred to a 96-well plate on ice followed by 2µl ExoSap-IT. The plate was vortexed and incubated at 37^{o}C for 15 minutes followed by 80^{o}C for 15 minutes to inactivate the enzymes.

7.2.3.3 Cycle-sequencing reactions

For the cycle sequencing reaction, the following was added to each sample: 9µl dH20, 3µl 5 x sequencing buffer, 1µl Universal forward primer and 2µl BigDyes v3.1. The reaction plate was placed on a thermal cycler and subjected to the following conditions: 96^{o}C for 1 minute then 25 cycles of 96^{o}C for 10 seconds, 50^{o}C for 5 minutes and 60^{o}C for 4 minutes.

7.2.3.4 Precipitation and sequencing

PCR products were precipitated by adding 2µl 125mM EDTA, 2µl 3M sodium acetate and 50µl 100% ethanol to each sample. The plate was inverted several times to mix and left at room temperature for 15 minutes before being centrifuged at 2090g for 30 minutes. The supernatant was discarded and the pellets washed with 70% ethanol. The plate was then centrifuged at 1650g for 15 minutes and the supernatant discarded. The plate was allowed to air dry in the dark before 10µl HiDi formamide was added to each sample. Following a, 2 minute incubation at 95ºC, the samples were then sequenced on an ABI3170 Genetic Analyser (Applied Biosystems) with BigDye Terminator cycle sequencing chemistries (v3.1, Applied Biosystems). Sequences were directly compared to the revised Cambridge Reference Sequence for human mtDNA (Andrews et al., 1999) (GenBank Accession number: AC_000021.2) using SeqScape software (v2.1.1, Applied Biosystems).

7.2.4 Restriction Fragment Length Polymorphism (RFLP) to Determine mtDNA

Carry-over in Nuclear Transfer Embryos Level of donor zygote mtDNA carry-over was determined by last hot cycle PCR restriction fragment length polymorphism (RFLP) analysis. Separate assays were developed for each discriminatory mtDNA sequence variant identified and were performed as described previously (Taylor et al., 2003, McFarland et al., 2004), with modifications as listed in Supplementary Table 2a (Appendix 3).

7.2.4.1 Last hot cycle (LHC)-PCR

Fragments containing the sequence variants of interest were amplified by PCR using primers listed (see supplementary table 1) and a last hot cycle performed with 1µl forward and reverse primer (20µM), 0.25µl Amplitaq Gold DNA polymerase and 5µCi [α-32P]-dCTP (3,000 Ci/mmol). LHC-PCR conditions were: 94^{o}C for 5 minutes, the optimised annealing temperature for the specific primer pair for 2 minutes (see supplementary table 1) and 72^{o}C for 8 minutes. The radiolabelled DNA was precipitated by adding 2µl Pellet Paint™ Co-precipitant (Novagen), 50µl 7.5M ammonium acetate and 200µl 100% ethanol to the sample. The sample was left to precipitate at room temperature for a minimum time of 1 hour and was then centrifuged at 13,000rpm for 10mins. The supernatant was removed and the pellet washed with 70% ethanol. The

centrifugation was repeated and the supernatant removed. The pellet was then left to air dry at room temperature for ~10mins and the number of counts per second in the sample measured using a gamma counter. The pellet was then re-suspended in a volume of dH_20 to give 1000 counts in 17µl of radiolabelled sample.

7.2.4.2 Restriction digests of radiolabelled PCR products

Restriction digests were completed by adding 16.8µl radiolabelled PCR product to 0.2µl 100X BSA, 1µl assay-specific restriction enzyme and 2µl of the appropriate 10 x restriction enzyme buffer (New England Biolabs, UK). The digests were left at 37^{o}C overnight. An additional 0.5µl restriction enzyme was added to the digest the following morning and the reaction left at 37^{o}C for an additional 1 hour.

7.2.4.3 Polyacrylamide gel electrophoresis

Vertical 12% polyacrylamide gels were prepared by adding 12ml 40% acrylamide (29 acrylamide: 1 bis) to 4ml 10 x TBE and 24ml dH20. The acrylamide was polymerised by addition of 75µl 25% ammonium persulfate (APS) and 75µl TEMED (Sigma) and the solution applied between glass plates and allowed to set at room temperature. 3µl 10 x loading dye was added to the digested products and the restriction fragments electrophoresed in 1 x TBE at 100V for 1hr and then at 150V for an appropriate length of time to allow clear separation of the products. The gel was dried onto a support using a gel dryer at 66^{o}C for ~1.5 hours. The gel was then exposed to a PhosphoImager screen (Molecular Dynamics, Eugene, Oregon) and left at room temperature overnight. The Phosphoimager screen was then imaged using the STORM Phosphoimager (Molecular Dynamics) and analysis completed using ImageQuant software (Molecular Dynamics). Carry-over donor zygote mtDNA was calculated as the percentage of total mtDNA in the recipient embryo.

7.3 Results

7.3.1 Potential to manipulate zygotes vitrified using an open versus closed vitrification system

The ultimate purpose of this study was to determine the potential to manipulate zygotes which had been vitrified. This is extremely important as it is highly likely that if pronuclear transfer were to become available as a clinical treatment one day that it would be difficult to ensure that both the embryo donor and embryo recipient would both be ready to undergo egg retrieval on the same day. Vitrification will allow zygotes to be stored from both the oocyte donor and mitochondrial patient following fertilisation at the PN stage of development. Those zygotes which retained their pronuclei after the minimum 2 hour recovery period were used to determine the feasibility for use in pronuclear transfer procedures (please refer to 4.3**Materials and Methods** for the full PNT protocol). Those embryos which were successfully able to be manipulated were then cultured so as to determine potential for onward development *in vitro*.

7.3.2 Potential to manipulate zygotes vitrified using an open vitrification system

Here I initially explored the potential to manipulate zygotes vitrified using the open vitrification system ***(***Origio™). Unfortunately due to premature pronuclear breakdown (As covered previously in section 6.8) it was only possible to attempt manipulations for a small number of zygotes (n=25).

7.3.3 Differences in ability to manipulate zygotes following vitrification as opposed to fresh zygotes

This is the first known report which has attempted to manipulate s vitrified zygotes. All zygotes were given at least 2 hours to recover following warming before they were moved to manipulation dishes. Zygotes were then incubated within the cytoskeletal inhibitors for the same time fresh embryos would be incubated (15-30 minutes). Holes were made within the zona pellucida using a minimum number of laser pulses, no apparent differences were noted when performing this with the vitrified as opposed to

fresh zygotes. Upon introducing the biopsy pipette into the embryo, the cytoplasm of the zygote did move around more within the zona pellucida compared to what would normally be observed for fresh zygotes. This did make it more difficult to orientate the zygote in order to attempt to remove the pronuclei. The cytoplasm of the zygote did offer greater resistance when the biopsy pipette was introduced into the zygote. This subtle difference in the composition of the cytoplasm following vitrification did suggest that the vitrification process did slightly alter the physiological properties of the zygote however not enough to prevent the zygotes being manipulated. On several occasions it was possible to remove pronuclei from the zygotes intact despite the observed differences in the nature of the cytoplasm however it did make it slightly more difficult to remove excess cytoplasm from the karyoplasts once removed from the zygotes.

In all 20 zygotes (n=20/25) were able to be enucleated and the pronuclear karyoplasts transferred to enucleated fresh (nom-vitrified) abnormally fertilized human zygotes. From the 20 successful PNT transfers is was possible to perform, 15 of the embryos resulting from these transfers survived with only 5 embryos degenerating immediately or on the following day. The 15 embryos which survived were then cultured and development monitored.

7.3.4 Development of vitrified (using the open vitrification system) and manipulated zygotes

Development for those zygotes vitrified using the open vitrification system and then manipulated and cultured was good, with; 20% (n=3/15) <4-cell stage, 47% (n=7/15) the 4-8cell stage and 33% (n=5/15)>8cell stage (Figure7.16 and Table7.9). This was compared to control data gathered for development attained by fresh embryos which underwent PNT manipulations; 25% (n=24/89) <4-cell stage, 44% (n=37/89) the 4-8cell stage and 31% (n=28/89) >8cell stage. Despite the small sample size of the vitrified and manipulated development attained by those zygotes which survived the procedure demonstrated developmental potential similar to that achieved by fresh manipulated embryos.

However given the vast differences in the sample size in the vitrified and manipulated embryo group (n=15) compared to the sample size of the non-vitrified (fresh)

manipulated embryos (n=89) (Fig7.2) and (Table7.1) it is difficult to make unbiased comparisons accurately compare and contrast fairly. The discrepancies in sample size can be explained by both the variability and unpredictability in obtaining samples and the impact of the vitrification process, including pronuclear breakdown during warming and recovery, which all acted to significantly reduce the number of zygotes which could be used to attempt manipulations upon following vitrification and warming.

7.3.5 Potential to manipulate zygotes vitrified using a closed vitrification system

I also examined the potential to manipulate abnormally fertilized human zygotes vitrified using the closed vitrification system (Vitrolife). It was only possible to attempt manipulations with a small number of zygotes (n=6). This was due to issues regarding the number of zygotes which t pronuclei were present in following warming and adequate recovery time prior to attempting manipulating (~2hours). Given these constraints it was possible to remove intact PN karyoplasts from n=6 zygotes (as depicted in Fig7.1).

Those zygotes which were manipulated, had their own PN karyoplasts fused back with them (reciprocal transfer) before they were then returned to fresh culture dishes and development monitored. Several of the manipulated zygotes did go onto cleave (n=4/6) to at least the 2-cell stage (Fig7.2). However given the small sample size, it is difficult to extrapolate our findings from this study using the closed vitrification system and contrast them to data we previously obtained for the closed vitrification and fresh manipulation and culture experiments (control data)(Fig7.2 and Table7.1).

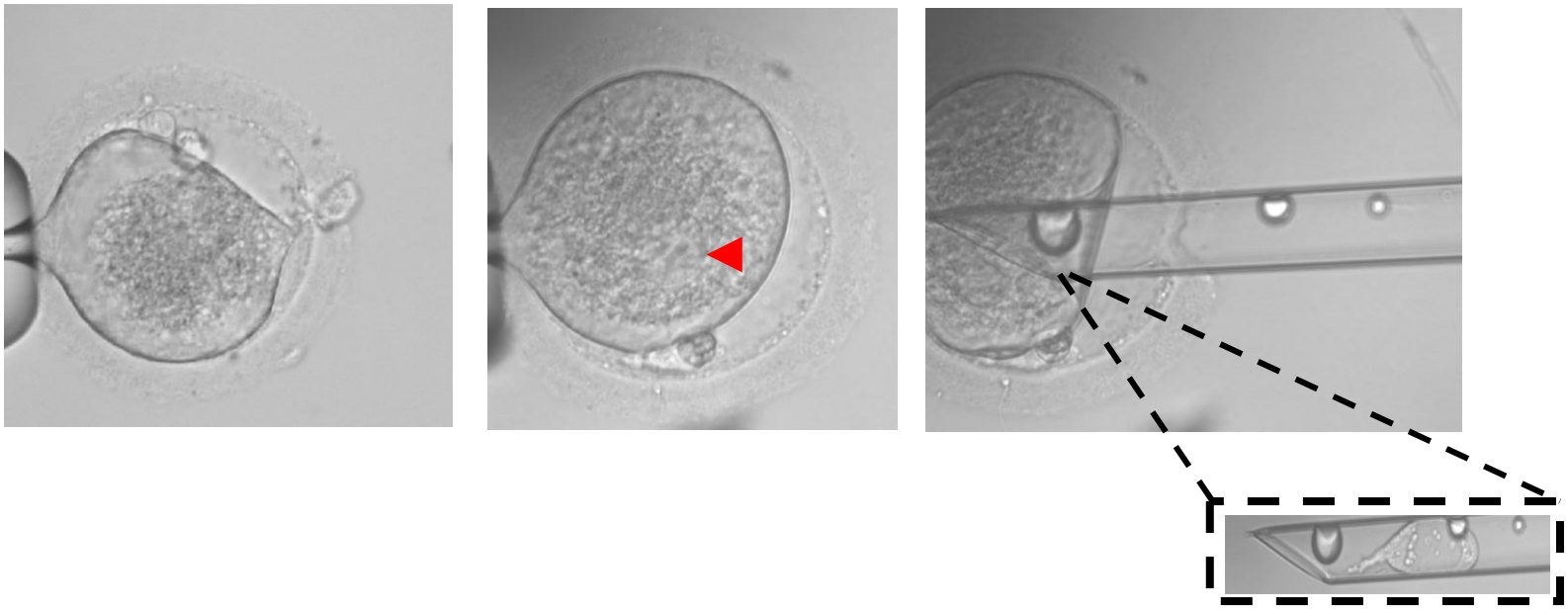


Fig7. 1 Manipulation of a vitrified and warmed zygote using (Vitrolife vitrification solutions and Rapid-i™) using a closed vitrification system.

Above images represent an abnormally fertilised zygote which was vitrified and then warmed using (Vitrolife™ RapidVit™ and RapidWarm™ vitrification solutions). Following ~2-4hrs adequate recovery time the zygote was still found to contain, a visible pronuclei (indicated by arrow) and transferred to a pre-prepared manipulation dish. The single pronucleus was able to be removed from the monopronucleate abnormally fertilised zygote successfully.

	<4-cell		4-8cell		>8-cell	
	Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)
Manipulated fresh	24/89	25	37/89	43.8	28/89	31.3
Vitrified and manipulated (open-device)	3/15	20.0	7/15	46.6	5/15	33.3
Vitrified and manipulated (closed-device)	2/6	33.3	2/6	33.3	2/6	33.3

Table7. 1 Comparison of development achieved for vitrified and manipulated, vitrified and cultured and fresh (non-vitrified) controls (abnormally fertilised human zygotes).

Above figure represents comparison in rates of development in freshly cultured embryos (blue) vitrified and cultured embryos (red) and vitrified, manipulated and cultured embryos (green) using an open vitrification system (McGill cryoleaf© as supplied by Origio™).

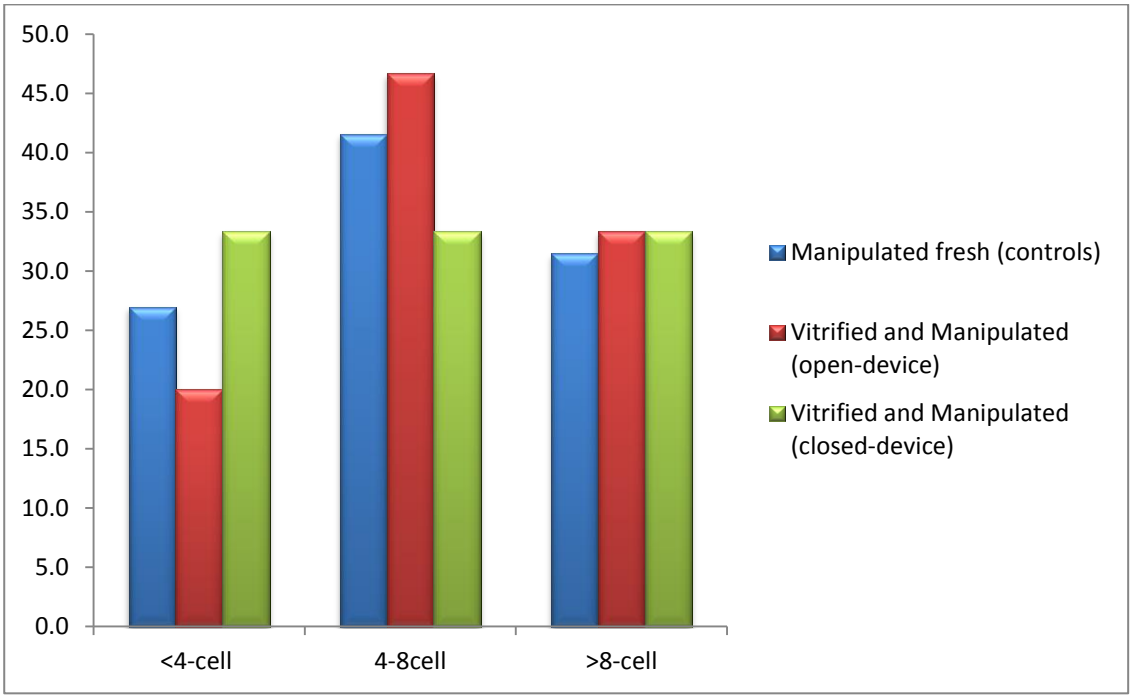


Fig7. 2 Comparison of development achieved for vitrified and manipulated, vitrified and cultured and fresh (non-vitrified) controls (abnormally fertilised human zygotes).

Figure represents development stages attained by fresh- vitrified manipulation and manipulated zygotes using the open vitrification system (red) fresh-vitrified manipulated using the closed vitrification system (green) and non-vitrified manipulated fresh control zygotes. Several zygotes vitrified using the open vitrification system (red) and manipulated demonstrated compatibility with onward development; 20% (n=3/15) <4-cell stage, 47% (n=7/15) the 4-8cell stage and 33% (n=5/15)>8cell stage. This was compared to development obtained from zygotes originally vitrified using a closed vitrification system (green) which were manipulated and cultured to assess developmental potential, with ; 33.3% (n=2/6) developing to <4-cell stage, 33.3% (n=2/6) the 4-8cell stage and 33.3%(n=2/6)>8cell stage. Development obtained by vitrified zygotes which were manipulated was compared to development obtained by fresh (non-vitrified) manipulated zygotes (blue) 25% (n=24/89) <4-cell stage, 44% (n=37/89) the 4-8cell stage and31%(n=28/89) >8cell stage.

7.3.6 Mitochondrial DNA Carry-over in vitrified reconstituted Pronuclear Transfer Embryos

Experiments were completed to determine if the proportion of mtDNA carry-over varied between blastomeres obtained from reconstituted embryos following the transfer of pronuclei from vitrified zygotes (vitrified using the open vitrification system) to fresh enucleated (non-vitrified) zygotes. To do this, pronuclear transfer embryos were cultured up to day 3 and then disaggregated into individual blastomeres (refer to chapter 3) to determine the level of donor mtDNA in each cell. This analysis revealed that there was limited variation in the level of mtDNA carry-over between blastomeres obtained from reconstituted embryos (n=4), ranging from <1-4% (Fig7.4). This analysis also revealed that one embryo contained no detectable levels of mtDNA from the pronuclear donor embryo in any blastomere (n=3). The average mtDNA carry-over in all embryos analysed was 5.5% (n=5).

However increased levels of donor embryo mtDNA were determined for individual blastomeres (n=5) obtained from one single embryo. Resulting in its average mtDNA carry-over calculated as 13.4%. Here the mutation loads ranged from 10-20% for each of the five individual blastomeres which were able to analyse for this particular embryo (Table 7.2). However this was an embryo which I manipulated whilst still in the initial stages of learning the PNT technique, therefore this may have contributed to the higher levels of mtDNA carried over to the recipient zygote. As with further experience and time spent performing the PNT technique I was better able to reduce the size of the karyoplast removed, and importantly minimise the volume of cytoplasm removed together with the pronuclei (Figu7.4). This is encouraging and further demonstrates the potential of vitrification as a potential means by which to successfully store human zygotes for the purpose of the PNT project.

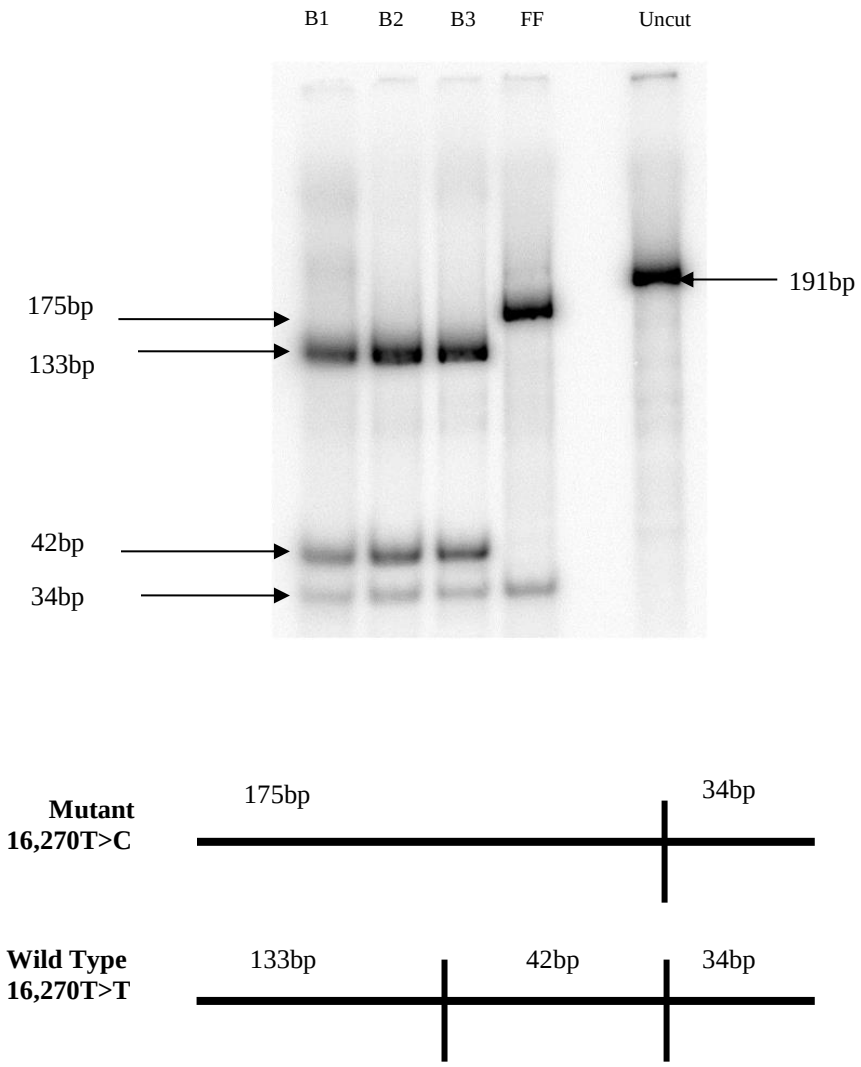
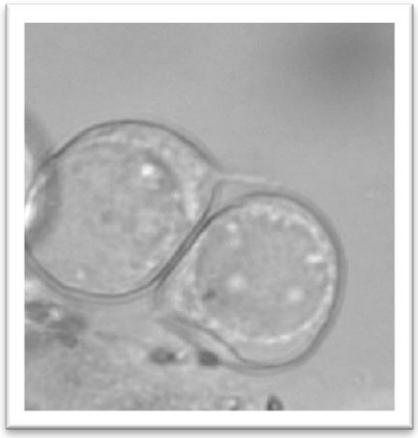
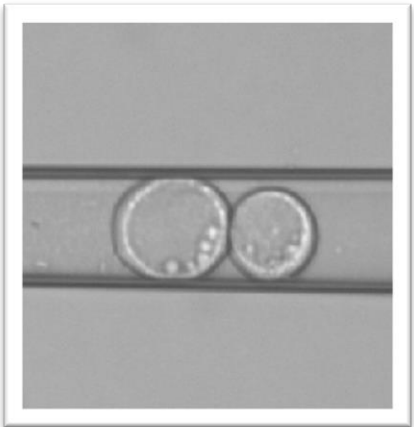


Fig7. 3 Mitochondrial DNA (mtDNA) carryover analysis (Last-hot cycle RFLP) of individual blastomeres disaggregated from vitrified pronuclear transfer embryos

Schematic diagram representation of the RFLP designed for the 16,270 mtDNA sequence variant identified following sequencing of follicular fluids obtained for the donor and recipient.



Karyoplasts prior to further manipulation to remove cytoplasm



Karyoplasts following further manipulation to remove cytoplasm

Development stage	Blastomere number					
	1	2	3	4	5	6
Three-cell	n.d.	n.d.	n.d.			
Five-cell	n.d.	3	3	2	3	
Four-cell	2	3	4	2		
Six-cell	n.d.	3	1	2	2	3
Five-cell	20	15	12	10	10	

Fig7. 4 andTable7. 2 Levels of donor mtDNA carry-over for 5 embryos vitrified and used for pronuclear transfer

(a)Images of pronuclear karyoplasts before and after further manipulation, so as to minimize the volume of cytoplasm carried over to the recipient zygote with the donor pronuclei. (b) Table represents levels of mtDNA carry-over determined for those embryos which were vitrified and warmed and following recovery used for PNT. Sequencing analysis was performed and single nucleotide sequence variants identified for each recipient and donor sample. RFLPs were accordingly designed following identification of sequence variants.

7.4 Discussion

This study is the first study to examine pronuclear transfer between vitrified and fresh abnormally fertilized human zygotes. Studies performed previously were successfully able to demonstrate the use of nuclear transfer techniques with the potential to prevent transmission of mtDNA disease in humans (Craven et al, 2010). These initial studies were performed using fresh abnormally fertilized human zygotes. Here they were able to demonstrate that these manipulated zygotes maintained potential for onward development and importantly the level of mtDNA carried over with the 'karyoplast' was low. This technique would ameliorate the risk that the mtDNA mutation would cause disease in the child resulting from the reconstituted zygote.

In the previous chapter I demonstrated that it is possible to vitrify abnormally fertilised human zygotes and upon warming and adequate recovery time perform pronuclear transfer between zygotes. In this chapter I set out to determine if indeed it was possible to manipulate using vitrified abnormally fertilized human zygotes initially. The second aim of this chapter was to determine if these embryos were compatible with development. Finally I set out to determine if mtDNA carryover was adversely affected by the vitrification procedure and would therefore make it not feasible to vitrify zygotes to be used for PNT.

I found there were several minor differences I observed when attempting manipulations upon the vitrified zygotes, which were not encountered when working with fresh zygotes. This included increased resistance to the biopsy pipette by the *zona pellucida*, indeed it is known that cryopreservation does induce hardening of the zona pellucida. I also found more difficult to manipulate the zygote. This we found slightly altered the ease at which the biopsy pipette was able to enter the zygote, resulting in a larger hole having to be made so as to not compromise the integrity of the zygote during the manipulation procedure. However it is quite possible that this may have been due to inadequate recovery time prior to attempting manipulations, which meant that the cytoplasm had still not yet fully recovered following warming. It is also possible that this may have been due to factors independent of the vitrification process, for example inadequate incubation within the cytoskeletal inhibitors prior to attempting manipulation procedures.

This study also demonstrated that not only is it possible to vitrify PN stage zygotes and successfully use them to perform PNT, they also demonstrate potential for onward development following manipulation. Development observed for zygotes vitrified using both the open and closed vitrification systems following manipulated was comparable to that observed for fresh manipulated control zygotes (33% versus 31% <8cell stage).

This is extremely encouraging as it demonstrates that it is feasible to use vitrified zygotes to perform PNT and the reconstituted zygotes are still compatible with development. However given the issues I encountered regarding PN breakdown the overall number of zygotes which I was able to attempt PNT with a small number of vitrified zygotes, therefore when performing the manipulations using zygotes vitrified with the open vitrification system I often used a fresh (non-vitrified) zygote as the recipient, to which the vitrified PN karyoplasts were fused back with to create the recipient zygote. This may have slightly altered outcomes. As the reconstituted zygotes contained fresh cytoplasm and only the vitrified 'nuclear' component. It is therefore possible that the cytoplasm helped to support development of the reconstituted zygote. This is consistent with the only other published report of attempts to manipulate vitrified non-human primate oocytes (Tachibana et al, 2012). This study failed to disclose the overall survival it was able to achieve using a device similar to the open vitrification device I used initially. They did state that using the particular cryopreservation method they opted for that development to the blastocyst stage was compromised for those cryopreserved oocytes which fertilized successfully following ICSI. However they did, none the less investigate the potential of spindle transfer (ST) between fresh and frozen-thawed non-human primate oocytes, for they which they examined fertilization and developmental potential. They noted that when fresh spindles were transplanted into vitrified cytoplasts, not only was fertilization following ICSI impaired to controls by almost half (50% versus 91%). Moreover all embryos in the vitrified ST group arrested development before reaching the blastocyst stage, whereas 57% of control embryos attained development to the blastocyst stage. However when they transplanted the vitrified spindle into a fresh cytoplast both fertilization (88%) and blastocyst (68%) formation were similar to those able to be achieved for fresh controls (91% and 57%).

It was also important to ascertain that mitochondrial DNA carryover was not affected by the vitrification process. Here I was able to examine mtDNA for individual blastomeres disaggregated from a number of fresh-vitrified PNT embryos. The levels of mtDNA carryover detected was generally low. Indeed non-detectable levels of mtDNA carryover were determined for some of the embryos I gathered from later into my experiments. This was extremely encouraging and demonstrated that vitrification was not exerting a detrimental effect on mtDNA carryover. This is encouraging as the average level of mtDNA carry-over detected in pronuclear transfer embryos reported by Craven et al (2010) were very low (ranging from <0.5 to 4.3%) and well below the level of mutated mtDNA associated with the onset of clinical disease (usually >60% of total mtDNA). However the levels of donor mtDNA detected in reconstituted embryos prior to improvements in my ability to manipulate karyoplasts before fusing with the recipient embryo were higher. Indeed the average level of mtDNA carryover detected for one embryo was ~13.4%, with one blastomere found to contain 20% mtDNA carryover. However higher levels of mtDNA carryover were also detected by L. Butterworth prior to improvements in performing the technique and manipulating the karyoplasts to minimize carryover (ranging from <0.5 to 25.3%).

It was extremely important to ensure that vitrification did not adversely affect the distribution of mitochondria in abnormally fertilised human embryos, as several studies have, demonstrated mitochondrial clustering around the pronuclei at the PN stage (Noto et al., 1993, Van Blerkom et al., 2000, Van Blerkom et al., 2002). If this were true we would expect to see high levels of mtDNA carry-over due to the increased numbers of mitochondria likely to be present within the pronuclear karyoplast. However, this was not observed in this study, which examined mitochondrial carryover in reconstituted embryos created by the transfer of pronuclei between fresh and vitrified embryos.

Furthermore unpublished work performed work has examined mitochondrial distribution in at the PN stage in both mouse and human zygotes and found mitochondria appeared uniformly distributed throughout the cytoplasm with no obvious mitochondrial aggregation around the pronuclei (unpublished data, L. Butterworth). However this same work did note that the distribution changed slightly in the presence of cytoskeletal inhibitors, with a clearing of cytoplasm observed in the cortical region almost immediately after exposure, however this did not result in an aggregation of mitochondria around the pronuclei.

Chapter 8 Final Discussion

Mitochondrial DNA is inherited through strict maternal inheritance and mutations in this genome are a common cause of inherited disease. However many mtDNA mutations are heteroplasmic and due to the genetic bottleneck during development, there is variable transmission to the offspring. It is therefore recommended that mothers with mitochondrial DNA mutations who want to have children should seek specialist advice from doctors who have experience of counselling specifically about mitochondrial genetic disorders. Indeed there are several different reproductive options available to these patients including; oocyte donation, preimplantation genetic diagnosis (PGD) and prenatal diagnosis (PND). This thesis therefore set out to examine the potential and feasibility of currently available IVF based techniques (PGD) aimed at minimizing the risk of transmission and those options currently being explored to prevent the transmission of mtDNA disease (PNT and MST) from mother to child.

Preimplantation genetic diagnosis (PGD) has only recently been recognized as a feasible option for women who carry heteroplasmic mtDNA mutations, due to the uncertainty surrounding mtDNA transmission and inheritance. The PGD technique involves the creation and identification of suitable embryos that have 'undetectable' or 'low levels' of mtDNA mutation and so are associated with a lower risk of the offspring developing severe disease. In recent years the technique has been successfully applied to a small number of maternally inherited mtDNA mutations (Steffan et al, 2006, Thorburn et al, 2009, Monnot et al, 2011, Treff et al, 2012, Sallevelt et al, 2013). Although experience is still limited and more information is required to determine the suitability of PGD for different pathogenic mtDNA mutations.

Studies conducted as part of this thesis were successfully able to perform mutation load analysis for a number of oocytes, cleavage and blastocyst stage embryos obtained from patients with six different mtDNA mutations (m.14709T>C, m.14487T>C, m.9176T>C, m.8993T>C, m.10158T>C and m.3688G>A). The results of which were encouraging and allowed for several important questions to be addressed. Firstly, does the mutation load observed in the biopsied blastomere accurately reflect the mutation load in the remaining cells of the embryo? Secondly does the mutation load observed at the point of blastomere biopsy remain consistent throughout embryonic development? Thirdly are there any apparent mutation specific differences regarding mtDNA segregation

within the same embryo? Finally, finally how does mutant mtDNA species segregate between embryonic and extra-embryonic cells within blastocyst stage embryos?

The results of my studies were in-line with the few previous which have revealed that mutation load is comparable between blastomeres (Thorburn et al, 2009, Treff et al, 2012), with interblastomere variations reported as 6% for the m.3243A>G mutation only (Monnot et al, 2011) and <10% for the m.3243A>G and m.8993T>G mutations (Sallevelt et al, 2013) in the majority of embryos examined. This is extremely important in order to guarantee the feasibility and efficiency of PGD for mtDNA inherited disorders. However, some variation in mutation load has been reported between blastomeres within a minority of embryos. This may occur more frequently in certain individuals, implying that genetic factors could be involved in mitochondrial segregation during embryogenesis (Vandewoestyne et al, 2012; Sallevelt et al, 2013). Therefore care must be taken when performing PGD assessing each case on an individual basis.

However although the mutation load determined in the biopsied blastomere appears to be representative of the mutation load in the remaining cells of the embryo, does this reflect the mutation load of the cells which will go on to give rise to the 'embryo proper'? Unfortunately there is currently very little information available regarding the segregation of mutant mtDNA between embryonic and extra-embryonic cells within blastocyst stage embryos. This study was able to provide an insight into mutation load segregation within the blastocyst, detecting similar mutation loads in cells origination from the ICM are highly comparable with those of the TE (for mutations m.14487T>C and m.3688G>A). There is one other single report which examined mutations loads from embryos carrying the m.3243A>G mutation, reporting a maximum variation of 9% mutant load between the ICM and TE (Treff et al, 2012). In addition, multiple biopsies from the ICM and TE of the same embryos revealed highly consistent heteroplasmy levels across the embryos, with mutation loads differing by 2-4% in the ICM and 3-11% in the TE (Treff et al, 2013). The low variation within the ICM supports the observation that mutant load remains relatively stable across fetal tissues (Matthews et al, 1994, Ferlin et al, 1997, Cardailoi et al, 2000, Monnot et al, 2010) and suggests that the mutant load determined at the time of biopsy will remain unchanged during embryo-fetal development.

The similarity in mutation loads observed in samples obtained from the TE and ICM also act to support the procedure of trophectoderm biopsy for mitochondrial PGD (Treff et al, 2012). Indeed there are many advantages of performing biopsy at this stage, including demonstration of developmental competency with embryos attaining development to the blastocyst stage, the increased number of cells acquired at point of biopsy for testing, less invasive procedure, and cells removed are not involved in contributing to the embryo proper so likelihood of compromising development is significantly reduced.

Another reproductive screening technique which has been proposed as an alternative reproductive screening approach is polar body biopsy. Polar body biopsy of mature oocytes prior to fertilisation avoids the need to create embryos for testing and is routinely performed for a number of nuclear-encoded genetic disorders (Munne et al, 1998; Wells et al, 2002; Magli et al, 2004; Montag et al, 2004; Kuliev et al, 2006; Mastenbroek et al, 2007). Its application in mtDNA disorders, however, has been disputed due to the apparent poor predictive value of the polar body in relation to the oocyte (Gigarel et al, 2011, Vandewoestyne et al, 2012). This was inferred from a study of over 50 oocytes obtained from patients with three different mtDNA mutations, as only 50% of polar bodies were reported to contain similar mutant loads (±10%) to the corresponding oocyte or embryo (Gigarel et al, 2011). The variation in mutant load observed between the polar body and oocyte appeared greater in polar bodies with higher levels of heteroplasmy (>60% mutation load) (Gigarel et al, 2011). My own data revealed a high correlation in mutant load between the oocyte and the first polar body for a very limited number of oocytes carrying the m.3688G>A and m.10158T>C mutations, supporting the observation that mutation load determined in the polar body corresponds to the mutant load in the oocyte at least for low levels of heteroplasmy. However, exceptions may still occur as reported in the m.3243A>G embryo that revealed mutation-free polar bodies but higher levels of mutation (up to >50%) within blastomeres of the cleavage stage embryo (Vandewoestyne et al, 2012). Therefore, polar body based PGD may be considered unreliable for mtDNA mutations irrespective of the mutant load.

In light of the current reproductive options available which can be used to minimize the likelihood of conceiving a child severely affected by a mtDNA defect there is still at present no way to completely preventing the transmission completely. However recently two nuclear transfer based techniques have been suggested as a means, by

which to prevent the transmission of mtDNA disease from mother to child (Tachibana et al, 2009; Craven et al, 2010). Both techniques involve the transfer of the nuclear component but at two comparatively different stages of development: Metaphase II (MII) spindle transfer (MST) utilises unfertilised metaphase II stage oocytes whilst pronuclear transfer (PNT) requires one cell zygotes (embryos). Indeed studies examining MST in the primate model have resulted in the birth of healthy live offspring, demonstrating the promise of this procedure (Tachibana et al 2009).

When introducing a new reproductive technique into IVF clinics it is critical that the technique is able to be reproduced by other operators. This has been demonstrated for the MST technique previously and as part of this study I was able to demonstrate the reproducibility of the PNT technique. Both potential for onward development and levels of mtDNA carryover were found to be similar to those previously attained in the initial proof of principle studies (Craven et al, 2010). All of these studies were performed in abnormally fertilised human zygotes (Craven et al, 2010).

However abnormally fertilized human zygotes are known demonstrate a limited capacity for development due to their abnormal chromosomal constitution (Herbert and Feenan, 2006). Therefore although current PNT experiments have been able to demonstrate the feasibility and potential of the PNT procedure using abnormally fertilized human zygotes in order to accurately assess development obtainable following PNT and address further concerns regarding the safety of the procedure it is imperative that studies are reproduced using normally fertilized zygotes (2PN).

Indeed following an amendment to the HFEA embryology act bill in late 2010 the creation human zygotes for the purpose of the mitochondrial study was permitted. The first altruistic donors were recruited for the PNT project in November 2010 and since a significant number of furtherer donors have been recruited. However the likelihood of two donors both being ready for egg retrieval on the same day was foreseen to be highly unlikely due to discrepancies in each individuals responses to the stimulation programme and adherence to the treatment plan they are given as altruistic donors. It is essential that there be a means by which to cryopreserve oocytes immediately following retrieval or following fertilization at the single cell zygote stage.

Embryo cryopreservation is a routine procedure performed within IVF laboratories globally allowing couples to store supernumerary embryos created from their fresh IVF cycle, which can be used to attempt frozen embryo transfer (FET) with. Slow freezing

was primarily used as the method used to cryopreserve embryos however due to advances in technology, the ultra-rapid freezing technique, known as vitrification has now replaced slow freezing. Vitrification is a much more efficient method of cryopreserving, less time consuming and promises high survival outcomes. This study also examined the potential of the vitrification for both oocytes and PN stage zygotes.

Cryopreservation at the PN stage has been demonstrated previously using both slow-freezing and more recently vitrification, with predominately good outcomes (Troup et al, 1991; Al-Hasani et al, 1986; Liebermann et al, 2002; Isachnko et a, 2005; Kuwayama et al, 2007). Indeed there is much interest in PN stage cryopreservation in Germany due to the laws which prevent embryo cryopreservation for those embryos which have undergone syngamy (Ludwig et al, 2000; Liebermann et al, 2002; Isachenko et al, 2004). However there are several stage specific limitations which restrict the suitability of vitrification at the pronucleate stage for the purposes of the PNT project. This is primarily due to the time which pronuclei remain visible for, before undergoing PN breakdown, given they are temporary structures and are therefore only present for a limited amount of time.

Indeed it is known from studies examining pronucleus formation following ICSI that extrusion of the second polar body occurs between 2-4 hours following injection, followed by the first appearance of pronuclei within 6-8 hours after fertilization (Nagy et al 1994, 1998). These studies also found that disappearance of pronuclei occurred in a synchronous manner, with PN breakdown occurring ~20 hours hpi. As fertilization checks are routinely performed between 16-18 hours following fertilization it is likely that some PN stage zygotes are on the cusp of undergoing PN breakdown, when the zygotes are transferred to research. However as the studies performed in these were performed using abnormally fertilized human zygotes, it is likely that this further complicated matters. As the appearance and disappearance of PN has been shown to vary somewhat in abnormally fertilized zygotes with up to a 10 hour delay between the appearance of the first and third pronuclei, which is not observed for normally fertilized zygotes (Nagy et al, 1994, 1998). This may help to explain why experiments performed as part of this study found that PN only remained visible long enough to perform PNT in a limited number of warmed zygotes. However it cannot be guaranteed that this phenomenon was simply due to timing issues associated with the PN stage, the use of abnormally fertilized zygotes or a side effect of the cryopreservation procedure and the cryoprotective chemicals employed.

Given the time constraints associated with PN appearance, it is essential that alternative stages of vitrification are explored, this includes oocyte vitrification. However cryopreservation of oocytes has proven elusive for the best part of the past decade due to the incredibly fragile physiological nature of the oocyte, rendering it vulnerable to cryodamage during the cryopreservation procedure (Gok et al, 1993; Chen et al, 2004; Boneti et al, 2011). However following the re-invention of vitrification it is now been made possible to successfully cryopreserve at this stage and achieve good overall survival. Indeed several studies have now demonstrated the high recovery and survival rates which can be achieved following vitrification at the MII stage (Lucena et al, 2006; Ciotti et al, 2009; Capalbo et al, 2010; Rienzi et al, 2010).

The criteria normally used to assess oocyte survival following warming is very limited involving assessment of morphology and developmental potential. However in the last few years the meiotic spindle has been investigated in an attempt to assess oocyte viability more accurately (Santis et al, 2005). The spindle is a highly delicate structure, which can be damaged as a result of the cryopreservation procedure. This is because the spindle becomes depolymerised during the vitrification process and subsequently requires adequate recovery time following warming to allow the spindle time to reassemble (Sathananthan et al, 1988; Aman et al, 1994; Wang et al, 2001; Larman et al, 2007; Gomes et al, 2008; Ciotti et al, 2009). Following the introduction of the optical system referred to as Polscope®, it is now possible to assess oocyte viability in living oocytes. This is piece of microscopy apparatus that uses polarized light to allow observation of highly ordered subcellular structures, such as the spindle microtubules in mammalian oocytes (Liu et al, 2000; Moon et al, 2003; Konc et al, 2004; Navarro et al, 2005; Rienzi et al, 2005; Santis et al, 2005; Hyun et al, 2007; Larman et al, 2007; Coticchio et al, 2010). The Polscope offers the unique advantage of being totally non-invasive, preserving oocyte viability (Keefe et al., 2003). The Polscope device was used in this study to examine for the presence of spindles in oocytes following warming, affirming the success of the vitrification procedure for oocytes.

However as a result of the cryopreservation procedure the zona pellucida hardens, therefore it is not possible to perform routine IVF insemination and ICSI must be used instead (Vanderzwalmen et al, 2003; Lucena et al, 2006; Ko et al, 2008). Oocyte vitrification followed by ICSI has been shown is not inferior to fresh insemination procedures, with regards to both fertilization and embryo development rates and has been reported following several similar independent studies (Kuleshova et al, 1999;

Yoon et al, 2003; Kuwayama et al, 2005; Rienzi et al, 2010). As part of this study a small number of vitrified and warmed oocytes were used to attempt ICSI, which did prove successful. This would therefore suggest that vitrification at the metaphase II stage followed by fertilization using ICSI upon warming may prove the best approach to bank samples obtained from altruistic egg donors. Given that this overcomes issues associated with vitrification at the PN stage and allows for great control with regards to experimental procedures.

This study set out to address issues regarding the reliability and efficiency of current reproductive based techniques used to minimize the risk of transmission of mtDNA mutations. However it also set out to investigate the reproducibility of new techniques, which have the potential to prevent the transmission of mtDNA mutations, which is not possible with current techniques. However there is still much work which must be conducted before such nuclear transfer based approaches can be fully translated into a clinical treatment. However if the nuclear transfer techniques are demonstrated to be safe, they will offer hope to those mtDNA patients, whom have exhausted all options currently available to allow them to conceive and carry a child genetically and biologically their own.

Future work

There is indeed an ever growing demand for PGD by mitochondrial patients. Consequently it provides a potential source of embryonic samples which allows for examination of mutation loads for different mtDNA inherited mutations. Work undertaken as part of this thesis has so far been able to examine mutation load segregation in various embryonic samples for six different mtDNA inherited mutations. However this only represents a tiny proportion of the currently reported known pathogenic mtDNA mutations. It would therefore prove interesting and important from both a scientific and clinical standpoint to gather mutation load segregation data for as many other mtDNA mutations as possible to help build a picture of how this differs. This will certainly determine the value of PGD for women that carry these mutations.

I think it would also prove important to analyse mtDNA mutations in individual cells of the blastocyst obtained from both the trophectoderm and the ICM. This would allow us to determine if the mutation load indeed differs between individual cells of the ICM (mosaic) in contrast to the mutation load determined for the homogenate trophectoderm population. Furthermore in light of recent proposals suggesting the importance of the ICM in initiation of the bottleneck it would be of interest to attempt to perform analysis for individual cells of the ICM. It would be important however to ensure that these cells were not contaminated by any other neighbouring cells which may lead to inaccurate determination of mutation load for that cell.

These studies would involve developing assays which would allow for efficient separation of cells. Current approaches used in the laboratory involve manually dissecting the ICM from the trophectoderm cells. This does allow for the ICM population to be distinguished from the trophectoderm cells however this does not ensure that the ICM population is completely dissociated from all trophectoderm cells. However other approaches have been reported including enzymatic digestion of non-specific adhered cells, followed by separation and these could be used if samples are available.

Given the work which has been conducted so far to examine the potential of pronuclear transfer for the prevention of transmission of mtDNA disease and recent events concerning the licensing and permitting of the technique for clinical purposes; the implementation of this technique now seems more a of a reality. Much work has yet

still to be undertaken to fully assess the safety of the technique and its efficacy and efficiency.

One of the primary areas optimisation is ensuring that embryos attain development to the blastocyst stage following PNT. Several different aspects of the PNT procedure require refinement in order to achieve this. Currently cytoskeletal inhibitors are used to allow for embryos to be manipulated safely. Cytoskeletal inhibitors relax the cytoskeleton allowing for the biopsy pipette to safely remove the pronuclei, however these do possess some cytotoxity and it therefore important that exposure to such compounds is minimized and those with low cytotoxity are utilised. This has involved investigating a range of cytoskeletal inhibitors to determine which poses the least harm to the embryo. It will also be important that if the PNT procedure is to be made available as a clinical treatment that efforts be made to source a cytoskeletal inhibitor which is of clinical grade. Further experiments also need to be conducted to determine the effects of the new cytoskeletal inhibitors and the effects this may have in turn on mtDNA carryover. Another aspect of the procedure which requires attention is the effect of the hole made in the *zona pellucida* to allow entry of the biopsy pipette. This is due to concern that this could be allowing premature hatching of blastocysts on day 5 as opposed to day 6, which consequently negatively affects blastocysts quality. Various modifications have been developed to try and overcome this issue including using smaller pipettes to remove the pronuclei (which subsequently do not compromise the integrity of pronuclear karyoplast), making a smaller hole and using a sharp ended pipette. However despite these interventions blastocysts continue to hatch on day 5, which has (L.Hyslop- unpublished data) may result in reduced blastocyst quality. Indeed analysis of fixed PNT embryos for several key cell type markers has demonstrated that many of these blastocysts contain lower cells counts which has been suggested could be a consequence of premature hatching. There are still some options to be explored which might allow this issue to be overcome including the use of a nanomaterial to patch up the hole made for entry of the biopsy pipette. Another possibility is the use of a currently commercially available compound known as 'embryo glue'. These are both viable options which need to be investigated.

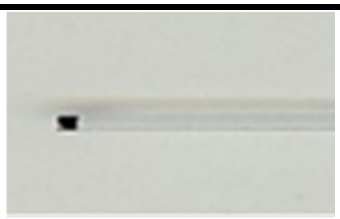
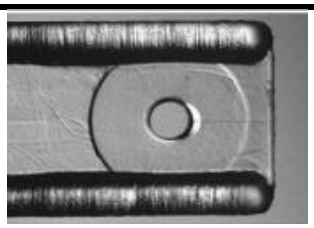
Further work is also required to be performed to examine the blastocysts which result from the PNT embryos. Following a report published by the scientific advisory committee (a panel of experts selected from relevant backgrounds to guide the decision making with regards to the awarding of a license to perform the PNT procedure when

safety and efficacy have been demonstrated) emphasis was placed on the need to ensure that blastocysts resulting following PNT embryos are not adversely affected. To address this issue a number of key lineage specific markers (Nanog, Gata6 and Cdx2) have been examined in the limited number of PNT embryos which attained development to the blastocyst stage in order to demonstrate that the PNT procedure does not affect lineage specification. The aim now therefore is to examine these markers and other useful cell lineage specific markers to fully examine the resulting blastocysts.

The scientific advisory committee also suggested that attempts be made to derive stem cells from the ICM cells of the PNT blastocysts. This would allow differentiation and proliferation to be examined. It was also suggested that mtDNA carryover resulting from the PNT transfer procedure should also be examined. It was therefore suggested that stem cells could be derived from the ICM cells of these PNT embryos and mtDNA carry-over examined via this method. This would also allow for mtDNA carryover to be examined at different cell passages to determine if it remained consistent.

Work covered as part of this thesis briefly touched upon the vitrification of oocytes. However work needs to be conducted to ensure that the meiotic spindle is not affected or disrupted by the vitrification process. This will involve using imaging based techniques and possibly comparative genome hybridization (CGH) to ensure that the spindle is not harmed as a result of the vitrification process. Karyotyping would also provide useful information on the chromosomal constitution.

Appendix 1

	Comparison of vitrification protocols	
	Open method (Origio™)	**Closed method (Vitrolife™)**
		
Cooling Rate	20,000°C/min	1,220°C/min
Warming Rate		7,700 °C/min
Temperature vitrification is performed at	Room temperature (18-25°C)	Performed on heated stage and solutions pre-warmed to 37°C (set point 42°C)
Are solutions buffered?	HEPES buffered to help maintain pH during vitrification and warming procedures	Solutions consist of a MOPs buffered medium to help maintain pH during vitrification and warming procedures
Cryoprotectants equilibration solutions	(7.5%)1,2-propanediol (PROH), ethylene glycol(EG)	ethylene glycol
Cryoprotectants in vitrification solution	(15%)1,2-propanediol (PROH), ethylene glycol(EG) and 0.5MSucrose	ethylene glycol(EG), propanediol(PROH), ficoll and sucrose
Number of solutions used to vitrify	Two-step protocol	Three-step protocol
Exposure time to vitrification (final solution)	60 seconds max exposure	30 seconds max exposure
Number of solutions included in the warming protocol	Five-step protocol	Four-step protocol
Time in initial warming solution	3 minutes	10-30 seconds
Initial temperature warming solution is equilibrated and held	37°C	37°C
Temperature remaining dilution steps are carried out at	Room Temperature	37°C

Comparison of the open and closed vitrification systems utilised as part of this study

Table illustrates the main differences between the 'open' and 'closed' vitrification systems used in this study.

Primer	Forward	Reverse	Sequencing	Annealing temperature
m.3688G>A	CAGGGTGAGCATCAAACTCAAA*	TTGTGATAAGGGTGGAGAGGTTAA	GCAGTGCGCCGATCA	62°C
m.9176T>C	TGACTATCCTAGAAATCGCTGTCG	TTGGTGGGTCATTATGTGTTGTC*	GCCTACGTTTTCACACT	62 °C
m.10158T>C	TACCACAACTCAACGGCTACA	AAGGAGGGCAATTTCTAGATCAA*	AACGGCTACATAGAAAAA	62 °C
m.8993T>G	TTACCACAAGGCACACCTACAC	CTAGGGTGGCGCTTCCAAT*	ATTCAACCAATAGCCC	62 °C
m.14709T>C	CACTCAACAGAAACAAAGCATACA*	TTAGGGGGTTAGTTTTGCGTATT	AACCACGACCAATGAT	59 °C
m.14487T>C	GCCATCGCTGTAGTATATCCAAAG	ATTCTGAATTTTGGGGGAGG*	AGTATATCCAAAGACAACCA	62 °C

Supplementary Table 1 Primer sequences and PCR conditions used for pyrosequencing

Pyrosequencing assay conditions used for mutation load analysis (* Denotes the biotinylated primer included within the assay)

mtDNA Sequence variant	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Annealing temperature	Restriction enzyme
m.16,270T>C	TGTAAAACGACGGCCAGTGCAATCA ACCCTCAACTAGCA	GTGGTCAAGGGACCCCTATC	61ºC	*BfaI*
m.16,298T>C	TGTAAAACGACGGCCAGTCAGCAAT TAACCCTCAACTATCA	CAGGAAACAGTCATGACCGGGACGAGAAGGG ATTTGAC	60ºC	*MseI*
m.16126T>C	TGTAAAACGACGGCCAGTTACATTA CTGCCAGCCACCA	CAGGAAACAGCTATGACCGTGGCTTTGGAGTT GCAGTT	60ºC	*HpyCH4V*
m.16519T>C	TGTAAAACGACGGCCAGTCAGTCAA ATCCCTTCTCGTC	GGGAACGTGTGGGCTATTTA	60ºC	*HaeIII*
m.497C>T	GTATGCACTTTTAACAGTCACC	GGGGTGTCTTTGGGGTTTGG	61ºC	*AciI*

Supplementary Table 2 (a) Last hot cycle-PCR RFLP assay conditions

Last hot cycle-PCR RFLP assay conditions for pronuclear transfer embryos (* Digests were performed overnight with 10U restriction enzyme).

mtDNA Sequence variant	Restriction enzyme	Product sizes (bp)		
		Uncut	Wild-type	Mutant
m.16,270T>C	*BfaI*	209	34 + 42 + 133	34 + 175
m.16,298T>C	*MseI*	191	25 + 166	25 + 80 + 86
m.16126T>C	*HpyCH4V*	199	33 + 166	33 + 47+ 119
m.16519T>C	*HaeIII*	223	91 + 132	31 + 60+ 132
m.497C>T	*AciI*	159	27 + 44 +88	44 +115

Supplementary Table 2 (a) Details of restriction digests performed for each of the Last hot cycle-PCR RFLP mtDNA sequence variant assays

Comprehensive list of restriction enzymes and estimated product sizes for each of the Last hot cycle-PCR RFLP assays

Last hot cycle-PCR RFLP

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