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Implementation of Clinical Assisted Reproduction Technologies for the Improvement of in vitro Production of Porcine Embryos: From IVF Clinic to Pig Farm

A thesis submitted to the University of Kent for the degree of

DOCTOR OF PHILOSOPHY

In the Faculty of Sciences

2016

Maryam Sadraie

School of Biosciences

No part of this thesis has been submitted in support of an application for any degree

or qualification of the University of Kent or any other University or institute of learning

Maryam Sadraie

March 2016

Acknowledgements

Firstly, I would like to express sincere gratitude to my supervisor Professor Darren Griffin for the continuous support during my PhD study and related research, for his patience, motivation, and immense knowledge. Darren has given me the opportunity to develop my scientific career. I could not have imagined having a better advisor and mentor for my PhD study.

Thank you to the TSB, JSR, and Guy's and St Thomas' Hospital for providing me with laboratory consumables. I would especially would like to thank Dr Alan Thornhill who gave access to Guy's hospital Reproductive Centre and research facilities. My sincere thanks also go to Dr Virginia Bolt, Dr Ilic Dusko and all the embryologists from Guy's hospital for their help, patience and support during my research. Without their precious support, it would not have been possible to conduct this research.

Thank you very much to all members of the Griffin lab both past and present, especially Dr Kara Turner and Dr Katie Fowler for their continued support during my PhD and writing this thesis. Also, my thanks go to Giuseppe Silvestri, Claudia Rathje, Becky O'Conner, Becki Gould, Ben Skinner, Mike Romanov and Pamela Lithgow. The completion of this project was not possible without the help of these people.

My special thanks go to my best friend and partner Babak Mobasheri for his loving and steady encouragement.

Finally, all my love and thanks to my parents Mehry and Mahmood Sadraie. You have continuously supported me financially and emotionally and gave me the opportunity to continue my education in the UK. I am extremely grateful.

Dedicated to my wonderful parents, Mahmood and Mehry

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IV Abbreviations

aCGH   Array comparative genomic hybridisation

AI   Artificial insemination

ART   Assisted reproductive technology

Anova   Analysis of variance

ASMA   Automated sperm morphology analysis

CASA   Computer assisted sperm analyser

CC_2 Time interval between t_3 and t_2

CC_3 Duration of second cell cycle

CCO   cumulus-oocyte complex

CMA_3 chromomycin A_3

CTC   Chlortetracycline

DHA   Docosahexaenoic acid

DPA   Docosapentaenoic acid

ECR   Embryo cleavage rate

$EEVA^T$ Early embryo viability assessment test

FISH   Fluorescence in situ hybridisation

FHB   Fetal heart beat

HEA   Human fertilisation and embryology authority

Hpi   Post insemination

ICM   Inner cell mass

ICSI   Intracytoplasmic sperm injection

IVF   In vitro fertilisation

IVP   In vitro production

LB   Live birth

NPBs   Nucleolar precursor bodies

NAR   Normal acrosome region

PB   Polar body

PBS   Phosphate Buffered Saline

PCR   Polymerase chain reaction

PCGD   Pre-conception genetic diagnosis

PFAs   Polyunsaturated fatty acid

Pff   Porcine follicular fluid

PGD   Preimplantation genetic diagnosis

PGS   Preimplantation genetic screening

PN   Pronuclear

ROS   Reacted oxidative stress

SNP   Single nucleotide polymorphism

T_2 First cleavage stage

T_3 Second cleavage stage

t_b Full blastocyst

t_{sb} Start of blastulation

TE   Trophectoderm

ZP   Zona Pellucida

V Abstract

The world population is predicted to rise from 7 to 9 billion in the next 30 years and per capita meat consumption is predicted to rise by 20% in this time. This places a demand on current food producers globally (particularly pork producers as 40% of global meat consumption is pig meat) that is not sustainable unless sufficient innovations are implemented. Livestock production also contributes 18% of the earth's global warming and this is also set to increase. Solving these problems necessitates producing increased amounts of meat from fewer animals in a shorter amount of time. UK companies lead the world in developing livestock with superior genetic traits that drive increased productivity through greater feed conversion efficiencies, improved disease resistance and greater fertility. Disseminating and applying these advances into herds around the world however presents unique problems. That is, for female line genetics, (male line genetics can be disseminated via sperm samples) producers are left with no other choice but to transport live animals for establishing nucleus farms overseas (e.g. In East and Southeast Asia). This can be expensive; energy consuming, environmentally unfriendly, and carries significant animal welfare and disease transmission concerns. One possible solution is to preserve and transport superior genetics in the form of preimplantation embryos (preferably pre-genotyped for sex and desirable production trait). To date however pig IVF and production (henceforth termed "IVP") has not been successfully implemented. The purpose of this thesis was to contribute to an ongoing effort to improve pig IVP through fundamental studies of porcine reproduction. Specifically, the work focussed on boar sperm production and on the human system (IVF clinic data) to provide clues as to the likely effects of embryo biopsy – an essential precursor to genotyping a preimplantation embryo as follows: The first aim was to produce a working classification system for boar sperm morphology and test the hypothesis that there are differences between high quality and poor quality boars. A number of hitherto unreported features of sperm morphology were established as significantly different in the poor-quality boar seen group. The second was to assess the effects of stimulants (e.g. caffeine and adenosine) on capacitation and fertilization rates and ask whether there was a correlation between capacitation and fertilization. Here, the utility of caffeine was established and correlations were observed between sperm morphology and capacitation rates. The third aim involved establishing whether novel markers of correct sperm chromatin packaging (CMA3 stain, nuclear organization, sperm aneuploidy) were indicative of reduced fertility in boars. Here a significant association between the poor-quality boars and level of CMA3 staining was observed indicating that this test may be implemented in the future as a means of identifying poor quality boars. No significant association with nuclear organization nor sperm aneuploidy was observed however. Finally, attention turned to human IVF data to test the hypothesis that embryo biopsy adversely affected subsequent embryo development. Using state of the art time lapse imaging no evidence was found to indicate that biopsy had an adverse effect in humans suggesting that, if performed correctly, this may also be the case in pigs. Taken together, the results provide evidence for the potential of significant advances in pig IVP by adapting protocols already commonplace in humans. Indeed, during the project, and in part because of it, IVP success rates in the laboratory increased dramatically.

1   Introduction

1.1   Reproduction in mammals

Sexual reproduction is defined as the ability of an organism to generate haploid gametes (oocyte or spermatozoa) by meiosis during gametogenesis (Thibault et al., 2001) Following fusion with another gamete of the opposite sex, this process includes the successive rounds of mitosis in order to generate a biochemically self-sustaining living form, itself capable of meiosis.

1.1.1   Mitosis

Mitosis is the most common form of cell division in most species and is the source of cellular growth and repair. During mitosis, two daughter cells with a diploid chromosome complement ensue with identical chromosomes and genetic materials as the parent cell. Each chromosome is copied during the S-phase (DNA synthesis and chromosome duplication step) to generate identical sister chromatids. Mitotic division occurs in somatic cells, and consists of several stages such as prophase as chromosomes begin to condense, then pro-metaphase followed by metaphase (middle stage) and anaphase. At anaphase stage, condensed chromosomes are transferred to the poles leading to telophase then cytokinesis (O'Connor et al, 2008)

1.1.2   Meiosis

Meiosis involves two cellular divisions that result in the generation of four genetically different haploid products arising from one diploid cell (Kleckner et al, 1996).

The first meiotic division (meiosis I) results in generating half the genetic complement of the mother cell, and is therefore called the reductional division. The second meiotic division (meiosis II) is described as equational division and this phase is similar to

mitosis. Cellular division in this way results in the inheritance of a random combination

of genes in the offspring, via the shuffling of maternal and paternal genomes by

random segregation, crossing over and random mating (Terasawa et al., 2007) The

prophase stage during the first meiotic division is sub-divided into four separate

stages such as: leptotene, zygotene, pachytene, diplotene and diakinesis (Kleckner,

1996,Roeder et al., 1997) .These prophase sub-divisions are demonstrated in figure

1.1, and figure 1.2, which indicates the meiotic process.
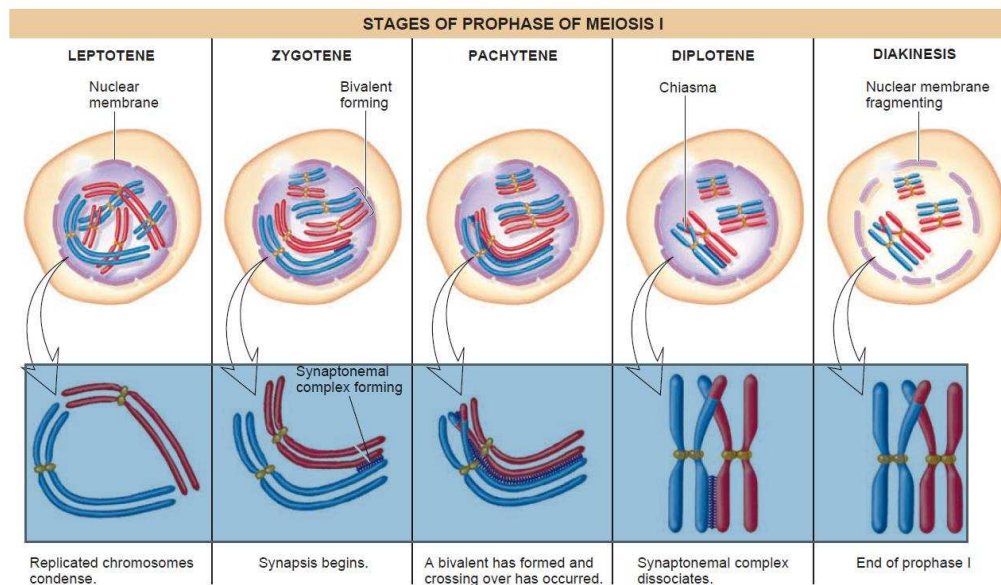
Figure 1.1. The stages of prophase I. Chromosomes start to condense in the Leptotene, then in the zygotene stage chromosome pairing occurred and synaptonemal complex formed in this stage. A bivalent has formed and crossing over occurred in the Pachytene stage. The chiasmata remain through the following stages of diplotene, and the synaptonemal complex starts to disappear at diplotene and chromosomes begin to align along the equator. Finally, chromosomes are fully aligned at the equator, the synaptonemal complex completely disappears and the nuclear envelop begins fragmentation. Image taken from https://www.quora.com/Genetics-and-Heredity.
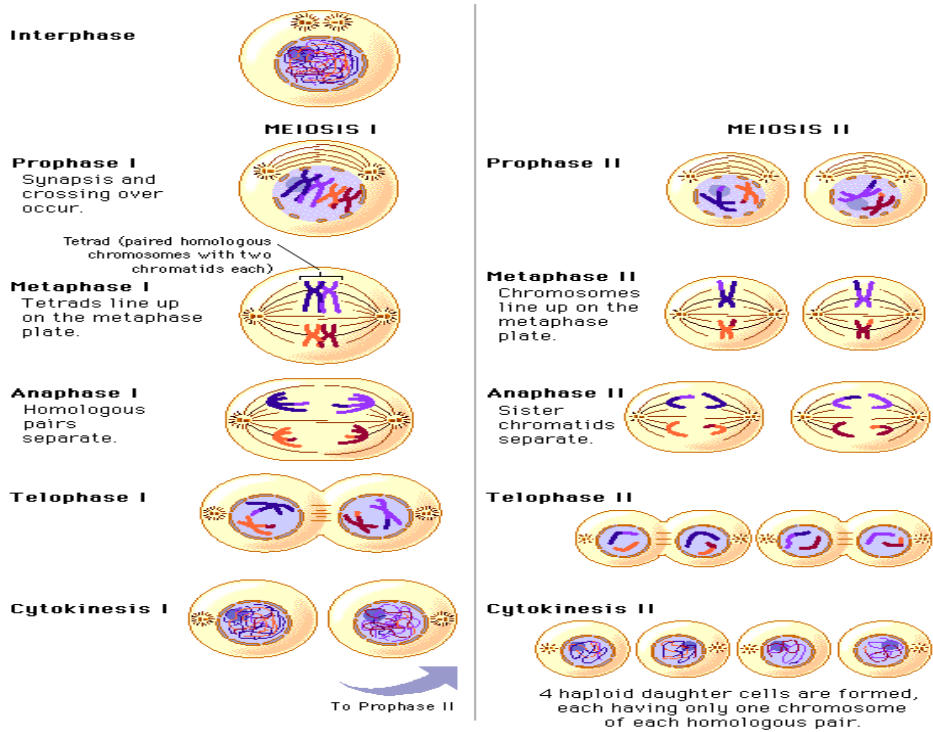
Figure 1.2. The whole meiotic process (meiosis I and II). During prophase I chromosomes begin to pair and decondense and the meiotic spindle starts to form. During metaphase I chromosomes align along the equator and attach to the spindle fibres. Then chromosomes are separated in the anaphase I stage. Homologous chromosomes are migrated to opposite poles in the telophase I stage, and two daughter cells are generated. Telophase I is the final stage of meiosis I and after that, meiosis II begins and it is quite similar to the meiosis I, while at the end of meiosis II, the telophase II stage, sister chromatids migrate to opposite poles resulting in four genetically different daughter cells. Image taken from https://staffweb.psdschools.org

1.1.3 Spermatogenesis

There are several distinguishable stages in the development of a mature sperm, such as the proliferative stage, the meiotic and the differentiation stages. In the proliferative stage, diploid spermatogonia go through mitotic divisions to generate diploid primary spermatocytes. Then primary spermatocytes develop into two secondary spermatocytes following meiosis I, and each of them then generate two haploid round spermatids after meiosis II. Round spermatids go through the differentiation stage and during this phase, involving the tight packaging of DNA, acrosome formation, elimination of excess cytoplasm and unnecessary organelles and formation of the

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sperm tail (Robles 2016, Clermont 1972; Russell et al., 1993). In males, the

production of gametes occurs continuously from the onset of puberty. Therefore,

meiosis I and II occur immediately successive to one another (Hilscher et al., 1974).

Figure 1.3 illustrates the stages that are involved in producing mature sperm during

spermatogenesis.
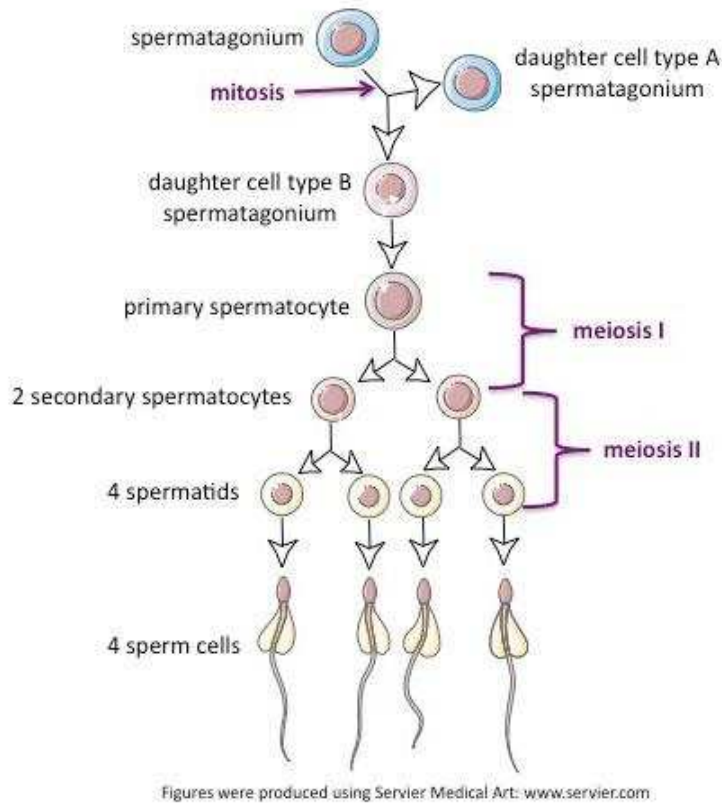
Figure 1.3. Schematic of spermatogenesis. This image was taken from
http://www.repropedia.org/spermatogenes

Before fertilisation, the sperm needs to undergo capacitation however this is covered in a

subsequent section (when discussing animal IVF).

1.1.4 Oogenesis

The female gamete is produced during oogenesis and it is a fundamentally complex procedure. Maturation of oocytes commences before birth as primordial germ cells undergo mitotic division to generate oogonia in weeks four to eight of gestation. During the next few months, the number of oogonia increases quickly, then the whole number of germ cells in the ovary reaches its maximum, approximately by seven million by the fifth month of prenatal development. At this period, cell death begins and many oogonia as well as primary oocytes degenerate. Therefore, by the seventh month most of oogonia have degenerated except a few of them located near the surface. All surviving primary oocytes have entered the prophase of meiosis I by this stage, and most of them are surrounded by a layer of follicular epithelial cells; the primary oocyte with the epithelial cells is called primordial follicle. Oocyte maturation continues at puberty, as near the time of birth all primary oocytes arrest at diplotene stage of prophase I at least until puberty. The whole number of primary oocytes at birth is estimated to be approximately 600000 to 800000. During childhood, most oocytes become atretic; nearly only 40000 remain by the beginning of the puberty, however only 500 of them will be ovulated. A group of growing follicles is found at puberty, so at the beginning of the puberty, each month 15-20 follicles chosen from this group of follicles to begin the maturation process, although most of these follicles die, while the remaining follicles start to build up fluid in the place known as antrum, thus they go through to the antral or vesicular stage. Fluid accumulation continue constantly prior to ovulation, follicles are swollen and are called mature vesicular follicles or Graafian follicles. The mature vesicular phase occurs 37 hours prior to ovulation. As oogenesis process resumes in the Graafian follicle and meiosis I is completed, two unequal size daughter cells are

formed. One of those cells is the secondary oocyte that retains most of the cytoplasm, and the other cell is polar body which receives none. The secondary oocyte immediately enters meiosis II, but it is arrested at metaphase II until fertilisation happens.

The process of ovulation occurs at around day 13, when the oocyte (now called the ovum) is evicted from the Graafian follicle with a complement of cumulus cells. When fertilisation takes place, meiosis II is completed (Ohno, 1962; Sadler et al., 2011)
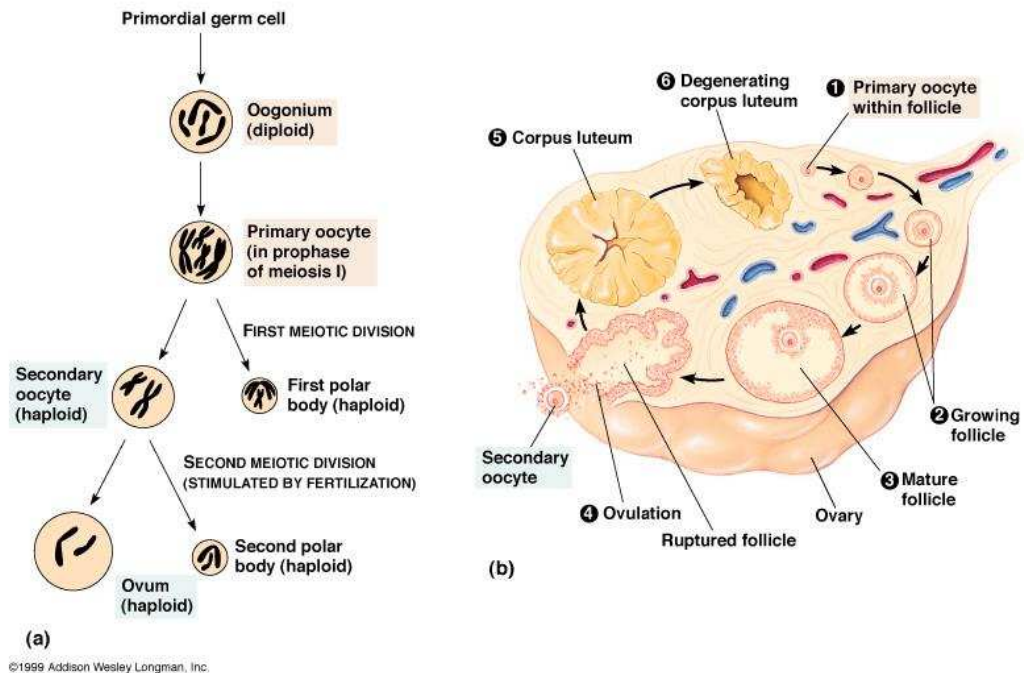
Figure 1.4. Oogenesis. Five stages are involved in the oogenesis process. First, epithelial germinal cells divide constantly to develop many diploid oogonia. In the next stage, oogonia grow to generate primary oocyte which surrounded by a layer of follicular cells. Then, oocytes go through the first meiotic division, but it arrests in meiosis I until puberty. After ovulation, meiosis I is completed, resulting in generating two unequal sizes of daughter cells. One cell is secondary oocyte and the other is polar body. The secondary oocytes enter meiosis II but it arrests at metaphase II stage until fertilisation happens. The image taken from the http://sharonap-cellrepro-p3.wikispaces.com/

1.2   Overview of fertilisation

The process of combining two gametes is known as fertilisation. This process occurs when mammalian eggs and sperm contact with each other in the female oviduct (Wassermann et al., 2001). Firstly, sperm with intact acrosome interact and attach to the zona pellucida (ZP),

and sperm go through the acrosome reaction or extracellular exocytosis. Five steps are involved in the fertilisation process: Step one occurs when mammalian sperm attractants the egg by a chemo attractant. During this procedure, heat-stable peptides are emitted by follicle cells surrounding the egg (known as chemotaxis) (Eisenbach et al, 1999). Step two happens when zona pellucida (ZP) attachment occurs. During this step the sperm undertakes the acrosome reaction, or cellular exocytosis. Step three begins when the spermatozoon penetrates the ZP. Next, step four occurs when the sperm binds to the plasma membrane in order to reach the privitelline space between the egg ZP and the plasma membrane. Finally, the fertilisation completes with, the appearance of fusion in step five. The action of this fusion event between the egg plasma membrane and a single spermatozoon prevents the egg plasma membrane from fusing with other spermatozoa that have penetrated the ZP. At this stage, the fertilised egg becomes a zygote, and as a result of this, other free swimming spermatozoa are not able to bind to the ZP. Figure 1.5 indicates a sperm with an intact acrosome binding to the zona pellucida (ZP) of the egg (Wassarman et al., 2001).
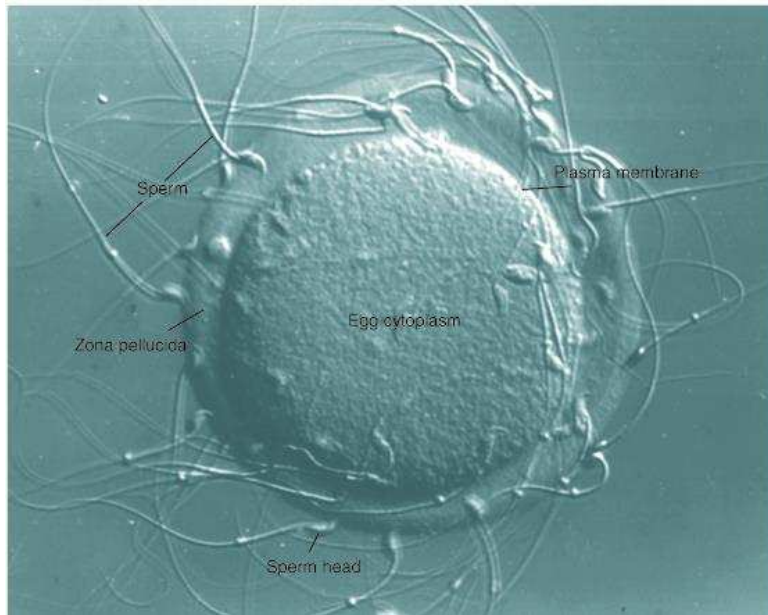
Figure 1.5. Binding of the sperm to the egg zona pellucida. Light photomicrograph of unfertilised mouse in vitro. The image taken from (Wassarman et al, 2001)

1.3   Preimplantation human embryo development

Human embryo development is a biological process which is still not well understood although remarkable similarities observed among other mammals, that is, all or most developmental stages observed in other mammals are distinguishable in humans (Niakan et al., 2012) The fertilised oocyte divides in two cells, followed by 4-cell and 8-cell stages known as cleavage stages. After that embryos undergo compaction process which the blastomeres maximise their contact with each other by adhesion, forming a compact ball that is held together by tight junctions. The next stage is morula (32 cell stage) that develops in to early and full blastocyst where inner cell mass and trophectoderm can be differentiated. The inner cell mass goes on to form the foetus and the trophectoderm goes on to form the placenta (Merton 2002, Cockburn et al, 2010). While the development of blastocyst progresses, cells in the two regions divide and the fluid cavity (blastocoel) enlarges. Figure 1.6 indicates the stages of human pre-implantation embryo development.
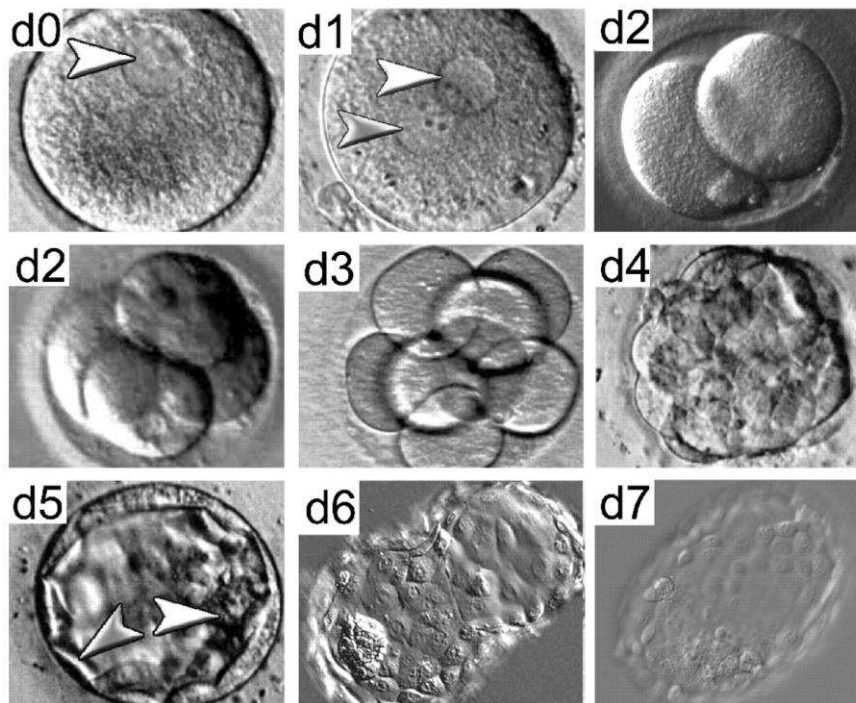
Figure 1.6. Stages of human pre-implantation embryo development. Images of human embryo development from day 0 to day 7 have been indicated. The arrow in day 0 and day 1 depicts pronuclei. Day 2 to day 3 consider as cleavage stage and around day 4, embryos begin to compact and then morula stage forms that consists of blastomeres in a compact cluster contained with ZP. Blastocyst forms on day 5 consider as a fluid-filled structure that consists of inner cells mass (it is indicated by white arrow in the image) and trophectoderm (it is indicated by gray arrow). On day 6 embryos go through hatching process which blastocyst hatches from the ZP, and embryo is ready to implant in uterine wall on day 7(Desai et al., 2000).

1.4   Infertility in humans

Infertility is defined as the inability to achieve pregnancy after one year of regular unprotected intercourse and affects approximately 15%-20% couples of reproductive ages (Allersma et al., 2013). Infertility can be indicated as male and female factor in adults and can have several causes such as environmental, infections, disease and genetic factors. For instance, the lack of sperm production in some men (azoospermia) (Carrell et al., 2008) and abnormal sperm morphological features are considered as important factors which

impact on fertilisation potential. Some clinical causes of infertility in women are associated to physiological predispositions, uterine/pelvis area abnormalities, blocked fallopian tubes, endometriosis, polycystic ovarian syndrome, premature ovarian failure, hormonal imbalances and lifestyle. It has been reported that fertilisation potential in both females and males decrease significantly in the last century, which can be associated with lifestyle options such as smoking, alcohol consumption, poor diet, drug abuse, infections and lack of exercise. The genetic factor can be considered as an important factor that affects fertility potential; genetic factors that can be involved in infertility may include single gene, polygenetic/multifactorial causes chromosomal abnormalities as discussed in section 1.1.3 and 1.1.4 (Tempest et al , 2004)

1.5   Origin of aneuploidy

1.5.1 Aneuploidy and infertility

Normal human beings have 23 pairs of chromosomes, although in several cases abnormal chromosome copy number can be observed, as chromosomal errors can happen at different stages of the development, which can affect the health of the developing embryo. For instance, in some cases patients have an extra copy of a particular chromosome like chromosome 21 that could cause pregnancy loss or Down syndrome, or the abnormality in chromosome copy number can be observed among sex chromosomes which can lead to pregnancy loss or Turner syndrome (Munné et al., 2004). It has been reported that chromosome abnormality can be considered as an important factor that contributes to decreased fertility in human (Wong 2008; Wilton 2005; Hassold et al., 2001).

Aneuploidy is referred to an abnormal number of chromosomes in a cell. For instance, having 45 or 47 chromosomes when 46 chromosomes are expected in human cells. When

whole chromosomes in meiosis I or chromatids in meiosis II and mitosis separate equally to opposite poles (disjunction), errors in chromosome separation (non-disjunction) is resulted in formation of abnormal chromosome numbers in the daughter cells. It has been reported that humans have the highest level of chromosomal aberrations among of any known animal species (Delhanty 1997, Daphnis 2005; Thomas 2003; Fragouli et al., 2008). These phenomena are still not well understood although various researches have been performed in this field. Previously, it has been reported that there is a correlation between chromosomal abnormalities in humans and infertility. Some of these common chromosomal abnormalities are known as trisomy (presence of one extra chromosome) of the sex chromosomes. Several factors impact on frequency of aneuploidy rate such as the maternal age, Y chromosome deletion, and different forms of translocations, inversions, increased rate of sperm disomy (Munné et al., 1998). Advanced maternal age has been introduced as a risk factor for forming chromosome number abnormality.

1.5.2 Advanced maternal age

Most chromosome abnormalities originate from female meiosis (mainly meiosis I) and it age-related reduction of meiotic recombination (Sherman et al., 1994).

The correlation between maternal age and aneuploidy was reported in 1930s for the first time (Penrose et al., 1933) and reviewed by Hassold in 2001 (Hassold et al., 2001). Hassold and colleagues suggested that the frequency of trisomy in advanced maternal age patients are as high as 15 times greater as females in their twenties. It has been suggested that chromosomal abnormalities, particularly trisomies, have been observed in in vitro fertilisation (IVF) for mothers above the age of 35 years. As maternal age impacts on the frequency of chromosome abnormalities, it leads to infertility problems, because older women are more likely to generate abnormal chromosome embryos which cannot develop to full term and aneuploidy causes pre-implantation embryo loss (Munné et al.,

1998). It has been reported that more than 40% of spontaneous abortions observed among women with advanced maternal age, and most of these spontaneous abortions result from embryos with trisomies (Robinson et al., 2001)

Van voorhis suggested that decreasing fertility in older females is mainly because of poor oocyte quality, as successful pregnancies increase significantly in advanced maternal age women when donor oocytes obtained from young females (Van Voorhis et al., 2007). It was previously reported that aneuploid oocytes indicate more poor quality, and there is a correlation between oocyte aneuploidy and advanced maternal age, mostly because of chromosome segregation errors in meiosis I (Hassold et al., 2001). As described in section 1.1.4, oocytes enter meiosis during embryonic development and they arrest in prophase I until puberty time, and then when ovulation occurs meiosis I is completed as homologous chromosomes are segregated, after that oocytes arrest again at metaphase II until fertilisation occurs. If fertilisation occurs, meiosis II is completed by separation of sister chromatids. It can be concluded that when primordial follicles activate to grow in the woman with advanced maternal age contains an oocyte which was arrested at prophase I for decades (Duncan et al., 2012). Several molecular mechanisms have been suggested to explain the meiotic origins of aneuploidy including errors in recombination, abnormal spindle formation, and microtubule-kinetochore interactions, and fault in the spindle assembly checkpoint reviewed by (Hunt et al., 2008). More recent findings in mouse suggest that deteriorating chromosome cohesion that occurs during the extended prophase I arrest is also likely a significant cause of age-associated aneuploidy reviewed in (Jessberger et al., 2012).

1.6   Assisted reproductive technology (ART)

Assisted reproductive technology includes all fertility treatments. This technology mainly belongs to the field of reproductive endocrinology (Coughlan et al., 2008) and includes in vitro fertilisation (IVF), intracytoplasmic sperm injection (ICSI), and embryo freezing (Glujovsky et al., 2012). Approximately 15%-20% couples of reproductive ages suffer from sub fertility in the UK, and nearly half of them will undertake some form of assisted reproductive treatments (Coughlan et al., 2008). The use of assisted reproductive technology has improved significantly and it is generally considered to be highly successful technique for the treatment of sub fertility (Shevell et al., 2005)

1.6.1   In vitro fertilisation (IVF)

The first successful human live birth from IVF was performed in the late 1970s (Steptoe et al., 1978). IVF is mostly applied to patients with advanced maternal age, repeated miscarriages and implantation failures (Thornhill et al., 2005).

In IVF treatment, the oocyte is fertilised outside the body and the embryo is cultured in vitro usually until the blastocyst stage when it is introduced to the uterus. IVF treatment occurs in three stages: First, Follicle Stimulating Hormone is used to stimulate the production of a higher number of oocytes than would naturally occur. During this treatment, vaginal ultrasound scans and blood tests are used to monitor the progress of ovulation. In the second stage oocyte collection is performed by using ultrasound guided aspiration of follicles using a needle. Finally, in the third stage, oocytes are combined with the semen sample in vitro and fertilisation is allowed to occur. The fertilised oocytes are then allowed to develop for 16-20 hours. The crucial part of in vitro fertilisation is stimulation of the ovaries in order to produce sufficient eggs to fertilise (Verhaak et al., 2007)
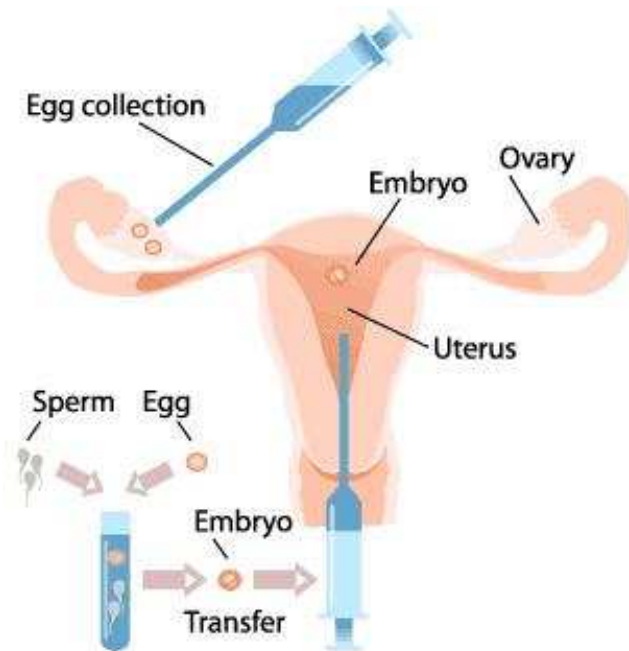
Figure 1.7. The main steps of IVF technique. Oocytes retrieved after hormonal stimulation are fertilised and incubated in vitro for a few days. The resulting hatching blastocyst stage embryo(s) can be transferred in utero image taken from http://www.nowpublic.com/health/ivf-diagram.

1.6.2 Intracytoplasmic sperm injection (ICSI)

ICSI was introduced as a new reproductive technology in 1992 as treatment for male

factor infertility including low sperm count, low sperm motility, or acrosome dysfunction (or

a combination of these) (Hodes-Wertz 2012, Jakab et al., 2005). As illustrated in figure

1.8, ICSI involves the use of a small needle (called a micropipette) that is used to inject a

single sperm into the centre of the egg. A study by Hiraoka and colleagues (2014)

indicated injecting both the head and the tail shows no difference in results in comparison

to only injecting the head (Hiraoka et al., 2014). Morphology of the sperm is considered as

an important factor to improve pregnancy rates (Bartoov, 2003; Berkovitz, 2005;Berkovitz,

2006;Berkovitz, 2006;Hazout et al., 2006), and therefore ICSI includes the selection of

morphologically superior spermatozoa. This can be achieved within the limits of the

conventional ICSI inverted microscope (magnification of x400) (Bartoov et al., 2001),

however use of higher magnification and a differential interference contrast optic (x1500

optical magnification) has been shown to additionally allow researchers and embryologists

to select motile sperm with a morphologically normal nucleus and a normal nuclear

content.

Figure 1.8. Intracytoplasmic sperm injection (ICSI). A single sperm cell is sucked in to the end of the needle. Then, this needle penetrated the oocyte zona pellucida, and the sperm cell is injected in to the oocyte to fertilise it. This image is taken from http://www.ivf.com/ivf_icsi.html.

1.7   Improving Assisted Reproductive Technology outcomes

The success of ART is essential for the selection of an embryo for transfer (Hardarson et

al., 2008). Single embryo transfer is considered to be important for decreasing the risk of

neonatal complications and pregnancy-related health problems, associated with multiple

pregnancies (Pickering et al., 1995). This is now standard practice in all UK IVF labs for

women under the age of 35 Precise criteria for early embryo assessment after in vitro

fertilization (IVF) is a main factor for selecting the best embryo to transfer to the uterus in

order to increase the implantation potential rate, successful IVF and high pregnancy rate

(Beuchat,2008;Ebner,2003;Gardner1998;Garello,1999;Gianaroli,2003;Murber, 2009;Kilani,

2009;Scott et al., 2007).There are several techniques which aim to promote single embryo

transfer to increase pregnancy rates; these include embryo morphology assessment, investigation of additional markers of viability (Racowsky et al., 2010) . Preimplantation genetic diagnosis (PGD) is a technique used in reproductive medicine for the diagnosis of single gene defects and chromosomal rearrangements like translocations prior to implantation (Frumkin et al., 2008) and preimplantation genetic screening (PGS) can be considered as a technique to select suitable embryo for transfer. PGS offers a chance to select the most chromosomally normal embryos as discussed in section (1.4) chromosome abnormalities, particularly aneuploidy considered as a factor risk to increase pregnancy loss. Embryo morphology assessment has been introduced as another appropriate technique to select high quality embryo for transfer (Racowsky et al., 2010).

1.7.1 Preimplantation genetic diagnosis (PGD)

As previously mentioned, the main aim of PGD is to produce a healthy baby that is unaffected by genetic disorders, therefore this technique is commonly performed in couples who are at risk of passing on a specific inherited disease (Harper 2012; Ehrich et al., 2012). PGD allows the selection and transfer of an unaffected embryo and it is recommended in patients at high risk of transmitting of a single gene disorder, in patients with advanced maternal age, repeated IVF failure (RIF), or in couples with recurrent miscarriages (Kanavakis, 2002; Ehrich, 2012; Ehrich, 2012; Ugajin, 2010; Lim et al., 2008). PGD also has application for gender selection. The first clinical PGD was performed in 1989 in couples who were at high risk of transmitting a sex-linked disease to their children (Handyside et al.,1990). Polymerase chain reaction was applied in order to identify a segment of the Y chromosome, therefore the absence of the band on gel indicated a female, unaffected embryo. Since this time, fluorescence in situ hybridisation (FISH) has been introduced in the early 1990s as a more robust method to perform embryo sexing over use of PCR to amplify a Y chromosome sequence (Griffin, 1994;Munné et al., 1993),however use of PCR

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continued for the detection of single gene disorders (Harper, 2010 Spits et al., 2009)

Furthermore, the use of FISH was extended with the emergence of probes for other

chromosomes in order to diagnose inherited chromosome abnormalities and aneuploidies

(Fridström, 2001; Mackie et al.,2002).

1.7.2 Preimplantation genetic screening (PGS)

PGS is a technique for the selection of chromosomally normal embryos with the best chance

of achieving a pregnancy and a live birth. In couples where infertility or sub-fertility is

diagnosed, PGS is recommended in order to improve the chances of a successful outcome.

Oocytes or embryos are selected following polar body, blastomere or TE biopsy and genetic

analysis ensues (Ehrich et al., 2012)

Despite the anticipated advantages of PGS however, it remains a controversial topic, and

many studies report no benefits of using the technique. Several studies carried out on

PGS at cleavage and blastocyst stage using FISH to examine aneuploidy have been

unable to show an increased delivery rate. Conversely, some of those studies indicated a

significant decrease in delivery rates, raising some concerns over the safety of PGS. In

addition, there is no evidence to suggest that routine PGS is beneficial for patients with

advanced maternal age (Staeesen2004; Mastenbroek, 2007; Meyer 2009;

Stevens, 2004; Debrock, 2010; Schoolcraft, 2009; Jansen, 2008; Harper et al., 2010)

Use of FISH in preimplantation genetic diagnosis

The main part of the FISH technique is designing an appropriate probe which is a small

piece of DNA of known sequence that is complementary on particular chromosome of

interest (Wilton et al., 2003). After labelling process, the probe is hybridised with a

metaphase or interphase cell and visualised under a florescence microscope (it is shown in

figure 1.9). When the FISH was tested on metaphase cell in order to make sure about

accuracy of technique, it can be performed on biopsied blastomeres in order to determine chromosome abnormalities. During this process, Biopsied blastomere cells should be fixed, the probes are added and after that the sample undergoes hybridisation process and incubation process and finally post hybridisation procedure and after that sample can be visualised under florescence microscope (Harper et al., 2012). FISH has been used for the controversial PGS and it is discussed in a later section.
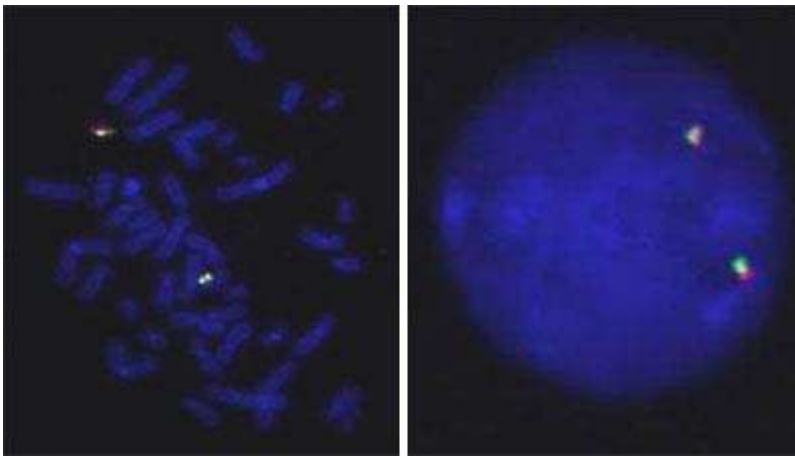
Figure 1.9. Dual colour FISH has been performed on normal human cell (lymphocyte). The left image is chromosome at metaphase and the right image represents an interphase nucleus. Image was taken from http://www.abnova.com/products

Array comparative genomic hybridisation (aCGH) is another important technique applied in both PGD and PGS. aCGH in basic principles is similar to FISH, but they have some differences. In FISH, normally up to five or six chromosomes are detected at a time, however the experiment may be repeated several times until all chromosomes have been detected, therefore this technique can be considered as a time-consuming process. While in the aCGH technique, all chromosomes are detected in one test, so it is very speedy technique (Ioannou & Griffin 2010), aCGH requires whole genome amplification of biopsied single cells, then fluorescence labelling process begins as the test DNA sample is mainly labelled green and

a reference DNA sample is labelled mostly red (De Ravel et al., 2007). These samples then go through a hybridisation process to choose spots of genomic fragments (an array), and the colour ratio is distinguished to identify copy number of whole chromosome or specific sequences within the test sample. So aCGH is applied for screening aneuploidy and recognition of deletions and duplications of specific genes in PGS (Fishel et al., 2010)

1.7.3 PGD limitations

Although PGD has many advantages as discussed in the previous section, while this technique has some disadvantages such as; technical, financial and ethical aspects (Kanavakis et al., 2002). Patients that are recommended to use PGD must first undergo IVF treatment, which can be a stressful and costly experience. This is added to with the costs and stresses involved in undertaking PGD (Harper et al., 2012). Secondly, as with any diagnostic test, there is a possibility that DNA contamination may have occurred during sample processing leading to false positive or false negative results (Kanavakis et al., 2002). Furthermore, there is a well-documented problem of chromosomal mosaicism(a situation in which different cells in the same individual have different numbers or arrangement of chromosomes) in developing embryos which makes clinical diagnosis more difficult (Kanavakis et al., 2002).With regards to FISH in particular, it is necessary to first spread nuclei on a glass slide, which results in the possibility of loss of nuclear integrity and morphology, and difficulty in interpreting results (Gutierrez-Mateo et al., 2004).

1.8 Embryo morphology assessment

Embryo morphology evaluation, as mentioned in section 1.7 is another tool that has impact on improving ART treatments. Embryo morphology assessment is done in two ways; traditional method by light microscope and the modern technique using time-lapse technology (see section 1.10). Pronuclear scoring according to Scott was based on three

morphological features including the size and location of the nuclei, the appearance of the cytoplasm, the numbers, size and distribution patterns of the nucleolar precursor bodies (NPBs) in the nuclei as described in figure 1.6 (Scott et al., 2000). According to this system, when NPBs are aligned at the pronuclear junction with equal number and size, they scored as Z1 (the best group). This section covers traditional ways to assess embryo morphology. Morphological features that are typically assessed in the analysis of embryo quality include: presence of multi-nucleation; the number, size and symmetry of blastomeres at cleavage stage; quality assessment of morula and blastocyst stage, as well as the degree of embryo fragmentation at each stage of development (Holte et al., 2007). In addition to reducing the number of embryos required for transfer, and in turn the risk of multiple pregnancy, embryo scoring in this way has provided an opportunity to evaluate several different culture media in order to optimise culture conditions, and allowing the opportunity to study embryo development in more detail (Ziebe, 1997; Sakkas et al., 1998). Currently however, embryo scoring systems differ among different laboratories, as each laboratory has its own embryo morphology assessment criteria. In the following section, however, the main procedures for embryo morphology evaluation which is used in the majority of IVF laboratories is summarised.

1.8.1 Morphological evaluation of zygote (16-18 hours post fertilisation)

Evaluating zygote or pronuclear quality is considered as the first step of embryo scoring. The fertilised zygote is categorised based on its morphological features, such as the number of pronuclei, the time of pronuclear appearance and disappearance, the size and alignment of pronuclei, and the size, symmetry and distribution of nucleoli. Scott and Smith (Scott et al., 1998) and Tesarik (Tesarik et al, 2000) were among the first to suggest systems for the assessment of pronuclear morphology. It should also be noted that this stage of evaluation

using traditional light microscopy is technically challenging and sometimes impossible, therefore use of a time-lapse device for scoring this stage is necessary (see section 1.10). Pronuclear scoring according to Scott was based on three morphological features including the size and location of the nuclei, the appearance of the cytoplasm, the numbers, size and distribution patterns of the nucleolar precursor bodies (NPBs) in the nuclei as described in figure 1.6 (Scott et al., 2000). According to this system, when NPBs are aligned at the pronuclear junction with equal number and size, they scored as Z1 (the best group). Z2 Zygotes are scored upon visualisation of equal number and size of nucleoli which are evenly spread into two nuclei. Alternatively, the zygote is scored as Z3 if unequal numbers or sized nucleoli are found in separate nuclei, or when the pronuclei are located at the periphery. If the pronuclei are scattered with unequal sizes, they were scored as Z4.

An alternative, more simplified classification model system was introduced subsequently by Brezinova (Brezinova et al., 2009). Here zygotes are scored according to the number, alignment and position of NPBs (Tesarik 2000; Łukaszuk, 2003; James, 2006 Brezinova et al., 2009). According to this scoring system, two different patterns (O or Other) are assigned based on pronuclei morphology of the zygote and early cleavage rate. Based on this system, pattern O is considered as a zygote with the same number of NPBs distributed in the nucleus or large NPBs distributed between two pronuclei. A zygote with unequal size of NPBs and non-symmetrical alignment was considered as Other (Brezinova et al., 2009). Wright and colleagues also indicated that PN morphology has a positive correlation with pregnancy and implantation rate, and additionally revealed that PN morphology was an accurate predictor of embryo developmental potential to blastocyst stage.
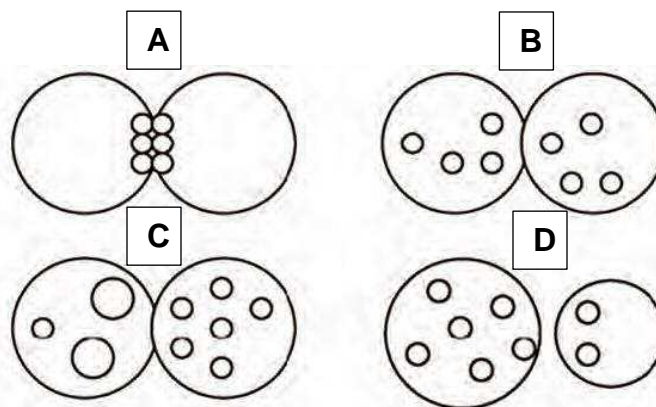
Figure 1.10. Zygote or pronuclear classification model according to Scott (Scott et al., 1998). Z1 zygote (equal numbers of nucleolar precursor bodies (NPBs) located at the pronuclear junction) (A), Z2 zygote (equal number and size of nucleoli which were spread equally in the two nuclei) (B), Z3 zygote (zygotes with unequal numbers or size of nucleoli in just one nucleus and equal number and size of nucleoli in another nucleus) (C) and Z4 zygote (the pronuclei are located in the periphery or are separated with different sizes) (D) (Nasiriet al., 2015)

1.8.2 Morphological assessment of cleavage stage embryo

The most commonly used criteria for selecting the best embryos for transfer have been cell number and morphology (Cummins et al., 1986). Many studies have been performed in order to establish an appropriate criterion to grade embryos at cleavage stage, however the most notable of these have been presented by Gerris (Gerris et al., 1999) and Van Royen (Van Royen et al., 1999). According to Gerris' established criteria, high quality embryos possess the following characteristics: four or five blastomeres on day two and at least seven blastomeres on day 3 after insemination, with less than 20% fragmentation rate on day 2 and absence of multinucleation on day 2 and day 3 post fertilisations (Gerris, 1999; Van Royen et al., 1999). Alternatively, another criterion for embryo scoring was established by Depa-Martynow (Depa-Martynow et al., 2008) their classification model was created to score day three embryos   based on four grades, A to D. Based on this grading system, grade A embryo is considered as an embryo with at least seven blastomeres (7-9

blastomeres) and with a maximum of 20% cytoplasmic fragmentation rate. Grade B embryos are considered as embryos with 7-9 blastomeres with more than 20% cytoplasmic fragmentation. Grade C embryos are identified as embryos with 4-6 blastomeres and more than 20% fragmentation and Grade D embryos are considered as a poor-quality embryo with 4-6 blastomeres and over 20% cytoplasmic fragmentation (Depa-Martynow et al., 2008).

Similarly, the embryo grading classification method introduced by Desai and colleagues was also based on cell number and cytoplasmic fragmentation rate, but additionally included observations on cytoplasmic pitting, compaction, symmetry of blastomeres, blastomere expansion and presence/absence of vacuoles. According to Desai, the cell number and fragmentation rate were the most important factor in predicting pregnancy outcomes (Desai et l., 2000). Several embryo grading criteria have been established since the mentioned early models, with various subtle changes. More recent studies have suggested that the amount of fragmentation, as determined by the percentage volume of the embryo that is occupied by fragmentation, may have less importance than the pattern of fragmentation, that is, the size and spatial distribution of fragments (Alikani et al., 2000). As such, a classification model established by Stensen and colleagues (2010) was based on the amount of fragmentation as well as blastomere size. This is illustrated in Table 1.1 (Stensen et al., 2010) On the other hand, however, Pelinck and colleagues argued in 2010 that in similarity to Scott's early system, the cleavage rate plays a more prominent role in determining quality evaluation of a pre-implantation embryo prior to transfer. According to their findings, the most optimal embryo morphological characteristic is identified as the presence of 4 cells on day 2, 8 cells on day 3, with less than 10% fragmentation and no presence of multinucleation on both day 2 and 3 (Pelinck et al., 2010).

Score	Description
3	Even or uneven blastomeres and with 10%-20% fragmentation
2	Even or uneven blastomeres with 20%-50% fragmentation
1	High fragmentation that prevents counting of blastomere numbers
0	Cleavage arrest or morphologically abnormal embryo

Table 1.1. Morphological grading and embryo classification according to fragmentation and blastomere size based on Stensen's grading system (Stensen et al., 2010).

1.8.3 Morphological evaluation of blastocyst stage

It has been reported that day 3 embryo morphology is inadequate for the accurate prediction of implantation rate (Graham et al., 2000). Nowadays, most IVF clinics transfer embryos at the blastocyst stage, and therefore the blastocyst scoring system established by Gardener is used to select the best embryos (Gardner et al., 2000). This is described in table 1.2 and is based on 6 stages of blastocyst development, such as the start of blastulation (formation of blastula from morula), emergence of the early blastocyst, emergence of the full blastocyst, emergence of the expanded blastocyst, the onset of hatching and finally the emergence of the hatched blastocyst.

Expansion grade	Description
1	Blastocyst development and stage status
2	Blastocoel cavity more than half the volume of the embryo
3	Full blastocyst, cavity completely filling the embryo
4	Expanded blastocyst, cavity larger than the embryo, with thinning of the shell
5	Hatching out of the shell
6	Entirely hatched out of the shell

Table 1.2. Blastocyst scoring according to blastocyst expansion grade (taken from (Gardner et al., 2000)

In addition, a separate grading system was established to score ICM (Inner cell mass) and TE (Trophectoderm) independently (Gardner et al., 2000), as described in table 1.3 and table 1.4. Previous research that provides insights into the correlation between ICM or TE morphology on implantation rate remains contradictory and therefore it is difficult to ascertain a true definitive conclusion. While Richter and colleagues found that a larger ICM was more predictive of implantation rate as no difference in TE cell number was found between implanting and non-implanting embryos (Richter et al., 2001). Ahlstrom and colleagues reported that TE morphology is the most important predictive of live birth outcome (Ahlström et al., 2011) in agreement with Ahlstrom, Thompson and colleagues suggested that TE morphology was able to predict live birth outcome (Thompson et al., 2013). Moreover, others have shown that TE morphology is correlated with aneuploidy in the embryo. A study of 194 euploid embryos and 238 aneuploidy embryos revealed a 2.5-fold increase in aneuploidy in embryos with poor trophectoderm morphology (Alfarawati et al., 2011).

ICM grade	ICM quality
A	Many cells, tightly packed
B	Several cells, loosely packed
C	Very few cells

Table 1.3. Summary of the morphological grading classification at blastocyst stage to score the inner cell mass (ICM) (Gardner, 2000; Nasiri et al., 2015)

TE grade	TE quality
A	Many cells, forming cohensive layer
B	Few cells with a loose epithelium
C	Very few large cells

Table 1.4. Summary of the morphological grading classification at blastocyst stage to score the trophoectoderm (TE) (Gardner, 2000; Nasiri et al., 2015)

To the best of my knowledge, there have been very few studies that have applied strict morphological criteria to ask the question of whether embryo biopsy adversely affects subsequent development.

1.9   Embryo biopsy

The removal of cells from an embryo for PGD or PGS is known as biopsy. In early studies, acid Tyrodes was used to make a hole in the zona pellucida and blastomeres were removed by aspiration. Nowadays however, it is more common to use a laser in order to produce a hole in the zona pellucida. Biopsy can be performed on three different stages such as on zygote (polar bodies biopsy), cleavage stage biopsy and trophectoderm biopsy at the blastocyst stage (Harper et al., 2012).

1.9.1 Polar body biopsy

Polar body (PB) biopsy is performed by aspiration for both first and second polar bodies. Performing polar body biopsy requires making a hole in the ZP to have access to perivitelline space and also a small bevelled micropipette is required to aspirate the PB slowly (Montag et al., 2009). It has been suggested that timing of the PB (polar body) procedure depends on whether first PB biopsy or second PB biopsy is required. For instance, for performing first PB biopsy, aspiration is performed shortly after oocyte retrieval, while second PB is required to perform aspiration simultaneously or sequentially, so the second PB is aspirated 6-14 hours post fertilisation (Strom et al., 1998). PBs derived for maturing oocyte and biopsied PB can be helpful in order to diagnosis genetic status, as first PB genetic status diagnosis can be performed before insemination and it is referred to as Pre-Conception Genetic Diagnosis (PCGD) (Verlinsky et al., 1990). PB biopsy has an advantage for couples who have ethical objections to embryo biopsy. Furthermore, embryo biopsy is prohibited in some parts of the world. PB biopsy can be useful for these groups (Fiorentino et al., 2008) However, PB biopsy has its own limitation. As PBs has different morphology, the first PB normally has crinkly appearance whereas the second PB is smooth, proper distinction can be difficult. In order to overcome this problem, sequential aspiration is often suggested (Sermon et al., 2002).

1.9.2 Cleavage stage biopsy

Hardy and colleagues (1990) were the first group to report that cleavage stage biopsy does not affect embryo development, as embryonic blastomeres are totipotent. Therefore, removing one or two cells do not impact on the ability of the remaining cells to differentiate into all the cell lineages which is essential for embryo development (Hardy et al., 1990). Cleavage stage biopsy is performed three days after fertilisation, when embryos are at 16 cells stage. According to previous study, the blastomere numbers are quite important at the

time of biopsy. For instance, if biopsy is performed at four cell stage, the ICM to TE cells may be altered additionally, if biopsy is carried out at compaction cell stage, cell removal will be very difficult and can result in cell lysis (DuMoulin et al., 1998).

Several techniques have been established for performing cleavage stage biopsy and all of them are require the making of a hole in the zona in order to access the perivitelline space. The most used common techniques are blastomere aspiration, alternative techniques such as extrusion technique and flow displacement have been established as well (Muggleton-Harris et al., 1995). There are some advantages to cleavage stage biopsy as performing biopsy on day three give adequate times for cell genetic diagnosis as embryo transferred can be performed at blastocyst stage (Tarín et al., 1993). Removing two cells from an eight-cell embryo may also cause damage to the embryo and affect implantation (Cohen et al., 2007). However, this has not yet been fully assessed. In brief, selecting the appropriate blastomere for biopsy can play an important role in the outcome of the biopsy, as the size, orientation, shape and volume of the blastomere can significantly impact on the outcome of the biopsy (Hardy et al., 1990).

1.9.3 Blastocyst biopsy

Blastocyst biopsy has been developed gradually, and it is now performed in one of two ways. Either a hole can be made on a day three embryos which is then left in culture, allowing some cells of the trophectoderm to herniate and enable biopsy on day five or six. Alternatively, the hole is made on the morning of day 5. This means that in contrast to making the hole on day three, it is guaranteed that the hole will be at the TE as opposed to risking that the hole will be made at the ICM. Since the ICM will form the foetus, it is considered to be safer to biopsy from the TE. Once the hole is made on day 5, the blastocyst is returned to culture for a few hours to allow herniation of the TE and then several cells are aspirated.

(Kokkali et al., 2005). The advantage of blastocyst biopsy is that it enables the removal of several cells and therefore an increased chance of an accurate diagnosis. However, the disadvantage of blastocyst biopsy is that since the embryo is transferred on day 6, there is a very limited time frame for diagnosis, compared to 24-48 hours diagnosis time at cleavage stage biopsy. This problem was solved by the introduction of vitrification, where embryos are frozen until a diagnosis can be made (Youssry et al., 2008).

1.9.4 Impact of embryo biopsy on embryo morphology

As embryo biopsy, has become commonplace in the IVF lab (Harper et al., 2010), several groups have investigated its impact on embryo development. Using conventional microscopy techniques, several groups have observed a reduction in embryo quality and developmental rate and potential (Malter 2004; Cohen 1991; Schmoll, 2003; Duncan et al., 2009). In the mouse model, blastomere biopsy has resulted in increased blastocyst contraction and expansion movements (Ugajin et al., 2010). With the emergence of time-lapse technology, one group has re-visited the impacts of biopsy on embryo morphokinetics. This study suggested that the duration of the cell stage at which biopsy was carried out was extensively delayed in biopsied embryos compared to non-biopsied embryos. However, there was no significant difference in the duration of compaction, morula and early blastocyst stage in the biopsied versus the non-biopsied group. In fact, it was revealed that the duration of blastocyst stage was shortened in the biopsied embryos (Kierkegaard et al., 2012).

1.10 Time lapse imaging

Morphological assessment of the embryo based on classical method of embryo evaluation (section 1.8.1, 1.8.2 and 1.8.3) requires removal from the incubator and examination outside of the controlled environment, exposing the embryo to sudden, potentially detrimental changes in essential parameters such as temperature, humidity and pH (Zhang et al., 2010). However, a revolutionary technique known as time-lapse monitoring overcomes the problems associated with traditional embryo morphology assessment in this way (Arav et al., 2008). Instead, a time-lapse device allows the live study of human embryos in real time and in more detail in comparison to classical methods (Meseguer et al., 2011). Time-lapse technology has been considered a non-invasive tool to select better quality embryos for transfer. Using a non-invasive method of morphology analysis reduces risk to the developing embryos and in so doing promotes a safer environment for the embryo to grow and develop (Montag et al., 2013)

However, the first time-lapse studies in the field of embryology were not performed until 1929 when Lewis and Gregory studied the development of rabbit embryos. With advancements in the field of human IVF, it was another 55 years before time-lapse imaging was extended to monitor human embryo development in 1984 (Edwards et al., 1984). Since then, modern day time-lapse devices have been rapidly developed, and time-lapse systems are now considered to be the ideal tool for the observation of living cells. Several studies have suggested that time-lapse systems are ideal for the study of the dynamic biological processes of embryo development, as they are capable of providing morphological, dynamic and quantitative timing data in a non-invasive environment (Wong et al., 2010).The maintenance of constant culture conditions and data evaluation has been improved not only by developing technological advances in new devices but also by improving culture media

alongside the development of bioinformatics. This section therefore covers a comparison between time-lapse devices that are currently used in a clinical setting and discusses whether time-lapse systems can be useful predictive markers of embryo development and morphological assessment. Several time-lapse devices are available for monitoring embryo development, including Tokai-hit, Primo-vision, Auxogyn-Eeva, Genea-Geri, Esco-Mirli® Time-lapse and Embryoscope. It is important to note that time-lapse devices vary quite significantly in their design; The Tokai-hit time-lapse device consists of an incubator which is built around an inverted microscope (Payne, 1997; Mio et al., 2008), whereas the Primo-vision has a microscope inside a conventional incubator (Pribenszky et al., 2010). Recently the Embryoscope time-lapse device has been developed further to include all the necessary items such as microscope and incubator integrated into a single piece of equipment (Cruz, 2011; María, 2012; Meseguer, 2012; Cruz et al., 2012). In all cases, several different time points are recorded in the time-lapse images. These include: Disappearance of PN, timing of the first cleavage and appearance of nuclei in each of the two blastomeres, timing of the second cleavage and finally re-appearance of nuclei in all blastomeres. Thus, the use of time-lapse devices has several advantages and disadvantages. These are outlined in the following sub-headings.

1.10.1 Incubation chambers built in to microscopes (Tokai-Hit)

Several companies such as Tokai-hit (Sanyo) from Japan designed incubation chambers built around an inverted microscope, and a black Plexiglas incubator box adjusted by an air temperature controller around the stage (Hardarson et al., 2002). This time-lapse imaging is analysed by image analysis software contains a small humidified three gas mixture in vitro culture chamber with a glass top and bottom placed over the culture dish. The time-lapse imaging is analysed by image analysis software. Images of each group are recorded sequentially in minimal light at intervals of 30 minutes throughout culture. The embryos were

moved out of the light field between recordings (Holm et al., 1998). The advantage of this device is the production of high image quality and flexibility.

1.10.2 Primo Vision time-lapse device

The Primo Vision (CryoInnovations, Hungary) is inserted in to a commercially available incubator (Pribenszky et al., 2010) which allows for constant culture conditions in an optimal environment (Hlinka et al., 2008). The Primo-Vision time-lapse system monitors embryos in real time throughout the whole embryo culture period. The Primo Vision is composed of a compact digital inverted microscope, placed inside an incubator and connected to a controlling unit that can be composed of up to 6 microscopes outside the incubator for continuous embryo monitoring. Primo Vision has Hoffmann contrast integration for improved image analysis, thus enabling the precise, non-invasive observation of embryo developmental dynamics (Gábor Vajta et al., 2012), from polar body extrusion, pronuclear formation, time points of cleavages, cleavage intervals, blastocoels pulsation and the exact identification of fragmentation and cleavage patterns (Pribenszky et al., 2010). It is composed of both a capture and analysis software, allowing embryologists to create personalised reports and videos about embryonic development to share with colleagues and patients. Primo Vision time-lapse also has the additional benefit of remote access which allows embryo evaluations to be performed outside of the laboratory, making online assessment with colleagues or other professionals possible (Gábor Vajta et al., 2012;Santos Filho et al., 2010). The culture dish designed for use with the Primo Vision machine (either 9 or 16 well) permits all embryos to be viewed in the 2.5mmx2.5mm exposure area of the Primo Vision microscope (Rienzi et al., 2011). The dishes are based on the well of the well (WOW) technology; embryos are placed in individual wells but they still benefit from group culturing.

1.10.3 Early embryo viability assessment test (EevaTM) time-lapse

The Early Embryo Viability Assessment Test (Eeva) time-lapse system (Auxygen) was created by scientists from the Stanford University, who indicated the first link between embryo development, details of cell division as well as the molecular health of the embryo. The Eeva time-lapse device is composed of a microscope, which fits in to most standard IVF incubators and also offers automatic dark-field image capture and cell division pursuing without involvement by the embryologist; there is also no extreme light exposure to the embryos. Eeva is equipped with a scope screen which fits on the outside of the incubator, allowing embryologists to observe the latest images of embryos without opening the incubator and disturbing the embryos in terms of temperature and PH. Eeva^{TM} analyses embryo development and cell division timing parameters to enable embryologists to predict the future viability of each embryo by day 2. The Eeva^{TM} station provides images and videos for each patient; all information can be easily reviewed and all images and videos from this device are downloadable (Chen 2012; Ingerslev 2012; Cruz et al., 2013). The main benefit of using Eeva time-lapse is that, this system has a sophisticated software which be able to select the best embryo automatically based on embryo morphology (Cruz et al., 2012)

1.10.4 Embryoscope Time-lapse device

The Embryoscope is known to be an advanced device used in a clinical setting and it was approved for clinical use in the European Union in June 2009. It is composed of high quality Hoffman modulation contrast optics that allow the observation of morphological features (Gábor Vajta et al., 2012). The time-lapse microscope is equipped with high quality Specific Leica optics, designed for red light at 635 nm and used to eliminate high energy light exposure (Azzarello et al., 2012). The system provides a safe culture environment while also providing images and videos every 20 minutes and videos for up to 72 embryos. It uses a tri-gas system to permit fast and accurate regulation of CO_2 and O_2 with minimal gas

consumption and direct heat transfer temperature control by individual wells. The

concentration of CO_2 and O_2 is recovered in less than 5 and 15 minutes respectively after

closing and there is a continuous circulation and purification of the air supply. The fact that

the Embryoscope utilises dry incubation without water pans eliminates problems with water

condensation and fungal growth on surfaces in high humidity (Azzarello 2012; Azzarello et

al., 2012). It is equipped with embryo viewer software that can annotate and compare

development of selected embryos (Meseguer et al., 2012). When comparing all three time-

lapse devices, the Embryoscope time-lapse system has been suggested as the best choice

to monitor embryo development, mainly because the incubator is built into the device

avoiding embryo exposure to a changing environment (Meseguer et al., 2012).The

Embryoscope device also permits embryologists to analyse embryo morphokinetics,

monitoring the dynamics of embryo development, in addition to traditional qualitative

morphological observations, referred to as morphokinetics, provides surplus information on

the development of individual embryos, and development in more detail when compared to

other non-embryoscope time lapse systems (Campbell et al., 2013). Moreover, another two

integrated time-lapse systems have been developed recently; Geri (Genea, Australia) and

Miri® time-lapse (Esco, Singapore). Both of these time-lapse devices can be installed in any

clinic; they are both small and compact and each chamber contains heated-lid which allows

for constant temperature regulation; additionally, Miri Time-lapse system has a larger

capacity (84 embryos) than any other time-lapse device. Table 1.5 summaries the technical

characteristics of these devices.

Time-lapse devices	Tokai-Hit	Primo-vision	Eeva™	Embryoscope™	Ger-Genea	Miri™
Integrated incubator	No	No	No	Yes	Yes	Yes
Optics	Bright field	Bright field	Dark field	Bright field	Bright field	Bright field
Frequency of images		Every 10 minute	Every 5 minutes	Every 15 minutes	Every 10 minutes	Every 5 minutes
Focal planes		1	1	7	7	7
Capacity of patients	1	1	1	6	6	6
Number of embryos		16 group cultures	12 group culture	12 individual culture		14 individual culture
Automatic diagnostic tool	No	No	Yes	No	No	No
Operator dependent		Yes	No	Yes	Yes	Yes
Time consuming analysis	Yes	Yes	Automatic	Yes	Yes	Yes
Selection algorithm		User defined	Yes	User defined	User defined	User defined

Table 1.5. Summary of individual time-lapse Characteristic: Tokai-hit, Primo-vision, EEva™, Embryoscope™, Ger-Genea, Miri™

1.10.5 Time-lapse devices comparison studies in embryo research

Time-lapse video cinematography was developed by Payne and colleagues in 1997 the

main aim of developing this new device was to overwhelm the restrictions of observing

embryos by conventional microscope. The key advantage of using cinematography was

producing high resolution, continuous imaging, and also with this system the different

components of the cell can be discriminated and screened during the recording period

(Payne, 1997; Payne et al., 1997). Nevertheless, the disadvantage of using this system

was limitation to an observation time of 17-20 hours. This study revealed formation of PN

in human oocytes which were obtained from ICSI. Nine years later, Mio and colleagues

developed more sophisticated device for time-lapse monitoring. This device was the

Tokai-hit (Japan). The major difference between this device and the one was developed

previously was maintain optimal culture conditions on the microscope stage for long

periods (Mio 2006; Mio et al., 2008). Standard interval of image in these devices was 2

minutes. These devices can be used for longer period compare to previous studied

device. In this study the morphological procedure of fertilisation, cleavage timing and

embryo behaviour during development was studied. The result of this study was similar to

recent finding by recent time-lapse devices. The main advantage of using Tokai-hit

(Japan) time-lapse device was the high image quality and flexibility make it suitable tool

for research purposes. Nevertheless, this device disadvantage is that it cannot maintain

the stability of the embryo culture environment (Hardarson et al., 2002) and using this

system is difficult and complicated to set up and time consuming to activate and only

handle single patient at a time, therefore is quite difficult to monitor large sample size of

embryos, and extensive exposure to light from the standard microscope theoretically can

damage embryo development. As a result of this outcome, this system has not been

suggested for clinical using IVF (Hegele-Hartung et al., 1991). Such systems have been

used only for research purposes. Few years later the Primo-vision system

(CryoInnovations, Hungary) was developed, in this device the microscope is inserted in to

a commercially available incubator (Pribenszky et al., 2010). This device is less flexible

than Tokai-hit but offers more stable culture condition. Another advantage of using this

device is its well-of-well (WOW) group culture dish which has a beneficial impact on

embryo development (Pribenszky et al., 2010). Fancsovits and colleagues compared in

2013 conventional dish with Primo culture dish and they suggested that embryos from

Primo culture dish reach significantly higher pregnancy rate, embryo quality is improved,

and also 12% higher clinical pregnancy rate compare to conventional dish (Vitrolife,2011).

Another advantage of this device is that the system is switched off entirely between image

gathering in order to prevent exposure to electromagnetic radiation, as it has been

suggested that electromagnetic radiant impact in vitro embryo development by varying the

speed of cleavages, gene expression and enzymatic activity of embryos of different

species to decrease embryo viability (Pribenszky et al., 2010). Primo-vision culture dish is

multi-well and this culture dish contains 9-16 wells. Embryos in this system are covered by

a drop of culture medium. The Primo Vision device monitor up to 16 embryos from the

same patient. This system uses low intensity green LED (550 nm) illumination and also

this device assesses the embryos up to 11 focal planes (Kovacs et al., 2014). There is

one camera for each culture dish, so for research purposes more embryos are required

and maximum can have 6 cameras. Massive incubator is required for monitoring more

embryos; therefore maximum 96 embryos can be monitored. Another time-lapse device is

Embryo early viability assessment from Auxygen Company (USA). The microscope is

inserted in to any standard incubator in this device the same as Primo-vision. This time-

lapse device uses dark field illumination to better outline the cell membranes (Eeva™,

2010). The big advantage of using this device its automatic software to analyse embryos. Its software automatically select suitable embryo for transfer (Wong et al., 2010). Wong and colleagues in 2010 developed a method in order to predict blastocyst formation by Eeva^{TM}; an algorithm was developed for automated tracking of cell divisions up to 4 cell stage. According to this group results, an automated prediction of blastocyst formation can be achieved as early as the 4-cell stage (Wong et al., 2010). As this system produces an automatic dark field image to analyse quantitative information on embryo development, some embryo morphological characterise like multinucleation is not possible to observe by dark field devices (Swain et al., 2013). More recent time-lapse device is the integrated system which is known as the Embryoscope (Unisense Fertilitech, Denmark). This device has been designed for clinical purposes. A main advantage of this device is its incubator because there is no need to remove embryo culture dish during embryo development as it is an integrated system, compare to previous time-lapse devices embryos will be maintained in very stable culture condition without opening the incubator for 5-6 days (Cruz et al., 2011). It can provide images of 72 individual embryos at a time. Embryos culture individually in microwells which are moved one by one in to the field of view, being in a good focus of the inbuilt microscope at each of the image acquisitions. The Embryscope has capacity of 6 of these dishes, so in total 72 embryos can be monitored by taking images every 15-20 minutes, and embryos can be evaluated in 7 focal planes in this system. This device used low intensity red LED illumination (635 nm) with <0.5 secendom per image light exposure. It has been suggested that compare to other time-lapse devices, Embryoscope seems to be the most user friendly, therefore better embryo selection based on kinetic parameters precise cleavage timing are considered as a considerable benefit of this system (Freour et al., 2012). All these mentioned time-lapse devices have different design and have different way of observation of embryos and all of

them have some limitations (Kovacs et al., 2014). For instance, In the Embryoscope time-lapse device , the culture dishes are hold by a tray and this tray under continuous movement put each embryo individually in to the field of view. While the tray is filled (72 embryos), it takes 20 minutes until the next image of given embryo is taken. This 20 minutes gap does not let embryologists to distinguish rapid changes precisely, for example, S1 (t2-tPNf) (time interval between two cell stages and pronuclear formation) last approximately <30-35 minutes (Kovacs et al., 2014). As a result of continuous movement. Electromagnetic effects, heat and unstable organic compounds released from the Embryoscope device, it seems that this system has the potential to apply adverse outcomes, although no such negative outcome has been reported so far (Kovacs et al., 2014). While, this technology allows the system to boost resolution, Primo-Vision system needs notably less regular image acquisitions because this device monitors maximum 16 embryos at the same time without moving them; thus, the exposure to light, electromagnetic effects are less than those with Embryoscope. However, the Primo Vision system does not provide the image resolution as good as Embryoscope blastomere membrane can be observed by Eeva time-lapse device more precise than other time-lapse devices because Eeva uses dark field illumination; so, divisions can be demonstrated precisely by this system but there is a disadvantage by using Eeva system which is it does not give enough information about intracellular morphology and has limited capability to follow embryos after 2 days, as cell number increases after that. By the Eeva system is more difficult to distinguish large fragments with blastomeres, which could affect accurate embryo selection. Embryos are exposed to considerably higher light by the dark field system compared to other time-lapse devices with bright field.

The EEVA device has software that can predict which embryo is most likely to reach blastocyst stage according to observations of early markers by day 2 of development (Conaghan et al., 2013).

1.10.6 Advantages of time-lapse systems

Time-lapse imaging systems offer many benefits compared to traditional time-point microscopy, as they are able to photographs of the embryos at defined intervals over a defined period of time. Captured images are then subsequently processed into a time-lapse sequence, thereby allowing morphological, dynamic and quantitative data to be recorded in a time-lapse sequence. Producing embryo images using this system provides intricate detail regarding embryo morphology and indicates the exact timing of different cleavage events. This provides a valuable tool in embryo selection thereby leading to single embryo transfer in order to avoid multiple pregnancies as mentioned before (Meseguer et al., 2011) Secondly, all time-lapse microscopy images are analysed using specifically designed software packages. This image capture software is invaluable for the automated analysis of images, enabling quantitative assessment of embryo development from time-lapse videos. Whole embryo and blastomere parameters such as; area, perimeter, diameter, degree of fragmentation and changes in these parameters over time, can all be monitored by such packages (Meseguer et al., 2012). Finally, and arguably most important, time-lapse imaging systems permit the analysis of embryo development in an optimal environment, which is maintained throughout the duration of the entire period of acquisition. Culture pH and temperature changes can cause a stress response and damage to the embryos and therefore many studies of time-lapse systems have highlighted that the maintenance of embryos in optimal culture conditions improves normal embryo development (Kierkegaard et al., 2012).

1.10.7 Disadvantages of time-lapse systems

The disadvantages associated with the use of time-lapse devices are largely attributed to technical challenges. These include the maintenance of samples in the culture dish, as well as ensuring that the embryos remain within the field of view of the microscope (Lemmen et al., 2008). Since embryos are non-adherent, there is the possibility they may move in culture dishes, particularly use of a motorised system to continuously move different embryos into the field of view during time lapse microscopy observations (Meseguer et al., 2011) alternatively, embryos may be cultured in micro wells within the culture dish. Secondly, time-lapse imaging introduces the potential for phototoxic effects on the embryos. Indeed, previous research has confirmed that light can cause DNA damage, localized heating as well as production of free radical species within blastomeres (Frigault et al., 2009). Time-lapse microscopy utilizes visible light in both bright field imaging and dark field imaging. However, light exposure in time-lapse microscopy is significantly less than that used in classical time-point analysis. Visible light is also less disruptive than high energy light such as fluorescence or ultraviolet light (Meseguer et al., 2011). In support of this, a previous study Sugimura and colleagues found that in both human and mammalian preimplantation embryos, time-lapse microscopy did not cause any considerable differences in the developmental potential of high quality embryos, blastocyst viability, blastocyst formation (Kierkegaard et al., 2012) or pregnancy rate (Sugimura et al., 2010),It is important to note however, that in some cases, not all events can be assessed in all embryos, as it is possible that some nuclei may be out of focus after the first cleavage (Lemmen 2008; Hlinka et al., 2012).

Time lapse application in IVF

Time-lapse technology allows embryologists to identify abnormal embryo cleavage which is not possible to observe using classical embryo evaluation methods. Abnormal cleavage is

defined by a direct or rapid cleavage to three cells in less than five hours (Rubio et al., 2012). This research group reported that the implantation rate will reduce when such embryos are transferred compared to embryos that did not display such abnormal behavior, and also confirmed that time-lapse devices have the ability to distinguish abnormal cleavage stage. There are numerous applications for time-lapse monitoring in the IVF laboratory. These applications include validation of conventional static assessment methodologies, prediction of embryo viability, investigation of the impact of variables such as culture media, O_2 concentration and drug regimens on embryo morphokinetics, investigation of embryo morphology and its impact on implantation and pregnancy rates, quality control and improving flexibility and working patterns. This section covers practical aspects of time-lapse imaging, developing embryo selection algorithms based on morphokinetic data and discusses how time-lapse systems can be integrated into the IVF laboratory to increase outcomes for patients.

1.10.8 Time-lapse methodology to select/deselect morphokinetic criteria

Time-lapse monitoring of embryo development, in addition to traditional qualitative morphological evaluations, provides morphokinetic information on individual embryos. These data are produced by the manual or automatic recording (annotation) of the embryo images, precise embryo developmental time is recorded and this timing can be analysed against outcome variables such as implantation, ploidy or live birth. This kind of analysis provides opportunity for embryologists to recognize the preferential selection for embryo transfer and cryopreservation. During embryo annotation by time-lapse system, if some embryos indicate very poor morphology according to defined criteria for morphological evaluation these embryos can be deselected for transfer.

1.10.9 Time-lapse algorithm designed for embryo selection

Time-lapse algorithms designed for embryo selection differ among different groups and type of time-lapse device (Aparicio, Cruz, & Meseguer, 2013). Parameters defined in each case are not precisely the same. Wong and colleagues in 2010 established an algorithm for Eeva^{TM} time-lapse device in order to have more appropriate embryo selection. The main aim of this group was to perform a large study to show that successful development to the blastocyst stage can be predicted by the 4-cell stage, before embryonic genome activation. According to this study three parameters introduced as an indicator of embryo implantation. These parameters are: duration of first cytokinesis, time period between the end of first mitosis and the beginning of the second mitosis and period between second and third mitosis. These parameters were validated by Eeva^{TM} time-lapse automated analysis. This group developed an algorithm for automated tracking of cell divisions up to 4 cell stage. This tracking algorithm utilises a probabilistic model estimation method according to Monte Carlo methods (Wong et al., 2010). Wang and colleagues (2013) developed another algorithm by Eeva time-lapse in order to recognise the number of cells at every time point of a time-lapse microscopy of early human embryo development. Authors in this study mentioned that the accurately measuring mentioned timing parameters in embryo development (Wong et al., 2010) requires an automated algorithm that can recognise number of cells at any stage during the time-lapse monitoring process. Three level classification method was used to categorise embryo stages (Wang, Moussavi, & Lorenzen, 2013). Another recent study by Conaghan and colleagues (2013) was performed to evaluate the first computer automated model in order to predict blastocyst formation and to establish how time-lapse screening data can aid the embryologist on day 3 embryo selection and transfer. According to this study, the probability of predicting high quality blastocyst is significantly increased by adding Eeva^{TM} with traditional day three embryo morphology and using this new technique

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alongside the existing embryo grading procedure improves day three embryo transfer (Conaghan et al., 2013). Primo-vision time-lapse device do not have automated tracking of cell divisions, while all parameters (PN formation and disappearance, 1st, 2nd, 3rd cleavages, multinucleation, fragmentation, compaction, morula and blastocyst) need to be entered manually. Pribenszky introduced first, second and third cleavage timing as an indicator for blastocyst formation in mouse embryos , Primo-vision time-lapse was used in this study (Pribenszky et al., 2010). The key findings using the Primo-vision time-lapse were the correlation between embryonic health and mitotic process, selecting better quality embryos compare to previous devices, Introducing non-invasive embryo cleavage rate (ECR) as a separate diagnostic method in order to provide an efficient detection of viable early embryos (Hlinka et al., 2008) .Meseguer and colleagues (2011) developed a multivariable model by Embryoscope time-lapse designed in order to classify embryos according to their probability of implantation rate. They designed an algorithm in order to select high quality embryo for transfer (Meseguer et al., 2011). The main difference between this study and previous designed algorithm by the Wong group was that in the Meseguer group investigation, all embryos were transferred and found morphokinetic parameters to predict embryo implantation but in Wong group study, none of embryos were transferred and their end point was blastocyst formation. Also, research was done only on cryopreserved embryos which could impact on research outcomes (Wong 2010; Meseguer et al., 2011). The Meseguer group also found a correlation between embryo morphological characteristics (blastomere evenness, fragmentation and multinucleation) and implantation in addition to cleavage timing (Meseguer et al., 2011). One year later, the Hashimoto group reported that embryos which spend shorter time to complete the second and third cell division, can lead to significantly better quality blastocysts (Kierkegaard et al., 2012). It has been confirmed that shorter cleavage time from 2 cell to 8 cell can lead to higher expanded

blastocyst and implantation rate (Basile et al., 2014). Kierkegaard and colleagues suggested that shorter second mitotic division <5hr can be considered as an indicator of predicting high quality blastocyst (Bronet et al., 2014), this finding was in accordance with result of (Wong et al, 2010), but Kierkegaard findings indicated that duration of second mitotic division predict only blastocyst formation not pregnancy. PN disappearance, multinucleation at 2 cell stage and appearance of nuclei after first division were not significantly different between implanted and non-implanted embryos (Bronet et al., 2014). This group finding was not in accordance with result of (Meseguer, 2011; Chawla et al., 2015). It can be concluded that the differences between these two group results can be as a result of sample size, as the Meseguer (2011) group used larger sample size; Kierkegaard group had only 84 embryos for their investigation. Campbell and her group designed a model to identify euploid embryos from aneuploidy. This group performed an experiment to identify whether morphokinetic parameters are different in aneuploidy and euploid embryos (Campbell et al., 2013). They performed this research on 64 embryos and all of these ICSI embryos were cultured in the embryoscope and were annotated, then embryos were biopsied on day 5 and preimplantation genetic screening (PGS) was done via array comparative genomic hybridisation in order to identify aneuploidy embryos. Campbell and colleagues designed an algorithm using non-invasive morphokinetic parameters to identify aneuploidy and euploid embryos. According to their model, embryos were classified in three groups of aneuploidy risk: low, medium and high risk. It was found that t_{sb} and t_b were significantly different between euploid and aneuploid embryos. These variables were used for classification of aneuploidy risk. According to Campbell (2013) model, if t_{sb} (start of blastulation) <96.2 hpi (post insemination) and t_b<122.9 hpi, identified as low risk of aneuploidy, if t_b (formation of full blastocyst) <122.9 hpi and t_{sb}≥96.2 hpi is in medium risk and embryo is classifies as high risk of aneuploidy if t_b≥122.9 hpi. It was the first published paper determining a correlation

between time-lapse morphokinetic parameters and embryo ploidy (Campbell et al., 2013). An earlier study indicated that there is a very weak correlation between blastocyst morphology and aneuploidy while this study was done without the benefit of time-lapse devices (Alfarawati et al., 2011). Campbell and colleagues performed another study to estimate the efficiency of their recent aneuploidy risk classification model. In this study, they evaluate their new developed model on patients who underwent ICSI treatment (88 blastocyst embryos transferred) without biopsy and pre-implantation genetic screening (PGS). The fetal heart beat (FHB) and live birth (LB) of transferred blastocysts compared based on calculated aneuploidy risk groups (low, medium, and high). It was found that there is a significant difference for FHB and LB rates between embryos categorised as low and medium risk of aneuploidy. This study introduced their developed aneuploidy risk classification model as an alternative method to increase live birth outcome when PGS is unavailable, although a larger data set is required to verify the efficiency of this model (Campbell et al., 2013). Ottolini and colleagues did not agree with the Campbell group conclusions, as they indicated that there was no difference in aneuploidy rate between fast developing blastocysts and slower developing ones. The Campbell group (2014) came to the conclusion that faster developing blastocysts have a lower aneuploidy rate and are therefore more likely to result in higher implantation rate compared to slower developing blastocysts. Also, the Ottolini group analysed 956 biopsied embryos and indicated that there is a positive association between maternal age and delay in blastocyst development (Capalbo unpublished data), so this group believed that the Campbell group designed aneuploidy risk model is predictive of maternal age alone. The previous group analysed embryos from maternal age range of 25-47 and therefore this large maternal range could lead to biased results (Ottolini, Rienzi, & Capalbo, 2014). However Campbell and colleagues in a published paper mentioned that ploidy was the main factor which impacts

on blastulation morphokinetics rather than maternal age (Campbell, Fishel, & Laegdsmand, 2014) performed a study to determine if Campbell (2013) aneuploidy risk classification models are predictive of aneuploidy/euploidy among all IVF clinics and this group suggested that time-lapse morphokinetic parameters do not approach the accuracy of preimplantation genetic screening (PGS) with array comparative genomic hybridisation. Another study was done by Basile and colleagues (2014) in order to select chromosomally normal embryos by time-lapse devices. They designed a larger study (504 embryos were analysed) than the Campbell group study. They also established another algorithm in order to select chromosomally normal embryos without performing PGS. According to their data which was provided by logistic regression, they established a hierarchical model and categorised embryos in four groups (A-D). This classification was made according to binary variables t_5-t_2 and cc_3. It was suggested that if the value of t_5-t_2 was inside the optimal range, the embryo was classified in group A or B. If the value of t_5-t_2 was outside the optimal range, the embryo was placed in group C or D, the classifying of embryos in A-D group according to cc_3 variable was similar to t_5-t_2. A significant decrease in the percentage of chromosomally normal embryos from group A-D was observed, so this new model can increase the probability of selecting chromosomally normal embryos without PGS (Basile et al., 2014). The main difference between this study and previous one with Campbell group was that in this recent research a very large data set was analysed and also chromosomally normal embryos can be identified by day 2 and may be this way can be considered as a tool to incubate embryos by day 3 instead of extending embryo culture to day 5. Also in this research day 3 biopsies were done but in the Campbell study day 5 biopsy was performed. A more recent study reported that the mean time duration for Pronuclei disapearance,t_2,t_3,t_4,t_5,t_5-t_2,cc_2 (duration of second cell cycle) and cc_3 (duration of third cell cycle) significantly differ between chromosomally normal and abnormal embryos, so this recent research introduced more

time-lapse variables in order to distinguish chromosomally normal and abnormal embryos (Chawla et al., 2015).

1.10.10   Time-lapse system, morphokinetics and treatment outcome

In the field of assisted reproduction time-lapse has been used for more than 40 years in both animals and humans (Payne, 1997; Mio, 2008; Wong, 2010; Nagy, 2003; Montag et al., 2011). Time-lapse technology results have indicated that the timing and coordination of events during early development is very important and has a positive correlation with embryo developmental potential and implantation. There are several studies in the literature that have focused on specific events during embryo development and how these may correlate with treatment outcome. To summaries, these have found that there is a correlation between PN morphology, appearance and disappearance on the timing of the first cleavage event, the number of blastomeres on day two and day three embryo quality (Payne 1997, Lemmen 2008). Following on from these observations, it was also found that the duration of the first cleavage (14.3 ± 6 minutes), the time period between the first and second mitotic division, the time period between second and third mitotic division, and cleavage synchronization were all accurate predictors of blastocyst formation and implantation rate (Wong et al., 2010). More specifically, it has been shown that embryos that cleave from two to three cells in less than 5 hours have statistically significantly lower implantation potential than embryos with a normal cell cycle length. Interestingly, the most abnormal short cell cycle (1.8 hours) was identified by Rubio and colleagues in 2012 was the strongest predictor of blastocyst formation are t_5 (five cell formation 48.8-56.6 hours) and cc_2 (time interval between t3 and t2) ≤ 11.5 h. Interestingly some research groups have also defined negative predictive factors correlated with limited implantation rate, including; direct cleavage from one cell to three cells stage, uneven blastomere size at two-cell stage and the presence of multinucleation at the four cell stage (Meseguer et al., 2011) by combining these findings

with positive factors an algorithm was established for embryo selection based on embryo morphokinetics and also embryos were classified into 10 groups overall however, not all studies are in agreement with one another and therefore the exact morphokinetic parameters which may result in a higher success rate are not clear-cut. For example, while one study found that early PN disappearance was associated with good embryo quality (Lemmen et al., 2008), others found that late disappearance of PN was associated with higher live birth rates (Azzarello et al., 2012), or that PN distribution was not associated with outcome (Mesequer 2011; Chamayou 2013; Kirkegaard et al., 2013). In addition, while one study showed that the timing of the first cleavage was associated with a positive outcome (Lemmen et al., 2008), another found that early cleavage was not associated with higher implantation rates (Mesequer et al., 2011). Similarly, some studies showed that the time to blastulation was indicative of implantations rate (Campbell et al., 2013) whereas others showed that that the time taken to morula compaction and blastocyst formation was not associated with outcome.

1.11 Assisted reproductive technologies in farm animals

After humans, the most common ART application has been observed in cattle and porcine. Cattle and pig IVF play an important role in industry as exporting breeds of each of them is very expensive presents the risk of diseases transmission, therefore by performing IVF on cattle or pig, many embryos have obtained and transporting embryos can be safer and cheaper (Mapletoft and Hasler et al.,2005). The second part of this thesis is focused on porcine IVF technology.

Pig breeding is of significant commercial importance, with worldwide figures suggesting that approximately 44% of total meat consumption (pork, beef and poultry) is pork, (United States

Census Bureau, 2012). The overall pork consumption increase has been largely stimulated by a remarkable growth of Chinese demand for meat while developed countries considerably maintained a steady trend (Bruinsma et al., 2003). The pig is an important biomedical model for human disease. There are numerous field of biomedical research that have benefited from the use of porcine models, for example research in areas such as cystic fibrosis (Rogers, et al., 2008), Parkinson's disease (Swanson et al., 2004), spinal muscle trophy obesity, female health, cardiovascular disease and nutritional studies reviewed in (Tumbleson and Schook et al., 1996) .First of all, some breeds have similar body size to humans allowing for an easy technology transfer. Secondly, the main advantage of preferring pig as a model organism in numerous biomedical researches is that the pigs reach sexual maturity earlier the other large animals and a good sized litter farrowed after a relatively short pregnancy. Finally, pig anatomy, genetics, pharmacokinetics and pathophysiology mimic those of the human body more precisely than other organisms such as rodents (Walters et al., 2012). Similarities are such that the pig can be a donor for xenotransplantation (Ekser et al., 2012). Also, availability of several well-defined cell lines, representing a broad range of tissues, will help with testing gene expression and drug susceptibility. Regarding genomics, the pig genome has high sequence and chromosome structure homology with humans, and pig gene sequencing technology has advanced significantly in order to assist with improving genetics and proteomics tools for pigs (Lunney et al., 2007). Pigs have been introduced as an accurate model of human embryo culture, as this part of research was mainly based on murine model. Several advantages that make pig as a suitable embryology models consist of that porcine oocytes can be easily retrieved from abattoir material, recent improvements have been achieved in their gamete/embryo manipulation in vitro (Nguyen et al., 2011). It has been suggested in comparison with murine, porcine and human embryos, porcine embryo has the most similarity with human embryos

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(Vajta et al., 2010). The comparisons are summarised in Table 1.6. The main difference between human and porcine embryos is that porcine oocyte/embryos have much more lipid than humans (McEvoy et al., 2010); this may suggest different metabolic patterns in early development. It has been suggested that high lipid content cause some difficulties in performing accurate microscopy and molecular assay as well as Cryopreservation (Sturmey et al., 2009).

1.11.1 ARTs in pig industry

ART technology has been introduced in section (1.6, 1.6.1 and 1.6.2). Common applications of this technology in farm animals industry include artificial insemination (AI) and in vitro fertilisation (IVF). These technologies in this section are discussed in pig industry.

1.11.1.1   AI

AI has been considered as the simplest of ARTs and the first one used in farm animal breeding. This technique is performed by injecting sperm in to female's uterus or cervix by an artificial tool such as a catheter in order to achieve a pregnancy in vivo by ways of other than sexual intercourse. AI has become a standard technique in pig production because it allows for increased selection potentials and servicing of a large number of females by most desirable sires (Roca et al., 2004). At least 70% of sows and gilts are mated in porcine industry in the United States, using the AI technique. Semen used for AI is normally collected from boars housed at off-site studs and semen is diluted in commercially available extenders, these extenders contain buffers and nutrients that preserve the spermatozoa viability for three or more days of post collection (Kuster at al., 1999).

1.11.1.2   Commercial application of pig IVF

As the demand for pork is increasing an efficient pig selection and breeding process is essential. The safety and quality of meat, animal welfare and environmental impact of breeding and transport process are paramount (Kenias et al., 2005). Application of pig IVF

technology in pig breeding industry is beneficial to the industry as this technology allows transporting embryos instead of piglets which reduces shipping cost, minimises the environmental impact of shipping and reduces animal welfare issues. Furthermore, embryos can be screened for required features such as gender (Sembon et al., 2008) and other genetic linked commercial characteristic like fertility and fat accumulation by using preimplantation genetic screening (PGS) methodologies (Foster et al., 2010) The commercial application of pig IVF and the advantages of pig as suitable biomedical model organism altogether attracted an interest in adopting IVF technique to the porcine model (Abeydeera, 2002; Gil et al., 2010).

Characteristic	Mouse Embryo	Pig Embryo	Human Embryo
Zygote's metabolic reserves	5-8 times smaller than humans	Similar to humans	-
Cytoplasmic lipid content	Low	Extremely high	Moderate
Development to blastocyst	Day 4-5	Day 5-6	Day 5-6
Embryo genome activation	2-cell stage	4-8-cell stage	4-8-cell stage
Amino acid metabolism	Different from humans	Similar to humans	-
Pyruvate/lactate versus glucose metabolism	Switches to glucose at 48h	No strict need for glucose until hatching	No strict need for glucose until hatching
Overall sensitivity in vitro	Low	Very high	High
Genome structure	Different from humans	Similar to humans	-
Methylation/ demethylation in early development	Extensive	Moderate	Probably moderate
Timing and location of embryo transfer	Exact match required	Flexible	Flexible
Developmental abnormalities after in vitro culture	Occasional	Very rare	Very rare

Table 1.6. Summary of the differences and similarities between human, pig and mouse embryos. Both mouse and pig have been introduced as a suitable model of human embryo research, while the pig appears to have more similarities with human and is therefore considered to be an accurate model in reproductive biology and embryo research. This table is adopted from (Vajta et al., 2010).

1.11.2 Porcine IVF challenges

Several research papers reported high live birth rates of IVF porcine embryos by using in vitro matured oocytes (IVM) (Yoshioka et al., 2003; Somfai et al. 2009), but porcine IVF is still faced with some techniques challenges such as polyspermy, oocyte maturation issues and fertilisation issues. This section covers these mentioned challenges

1.11.2.1   Polyspermy

The major unsolved issue in porcine IVF is polyspermy: penetration of two or sometimes more spermatozoa in to a single oocyte resulting in a polyploidy embryo. It has been reported that the incidence of polyspermy in vitro has been observed in approximately 50% of cases, which is significantly higher than the polyspermy frequency in vivo (Wang 1991; Abeydeera et al, 1997). Hunter and colleagues (1990) suggested that in vivo conditions, the oviduct microenvironment contributes in completion of oocytes to block polyspermic fertilisation (Hunter et al., 1990). Many approaches have been considered to decrease polyspermic rate in in vitro conditions including pre-incubation of spermatozoa or oocytes with cultured oviduct epithelial cells (Nagai et al., 1990) or follicle somatic cells (Wang et al., 1992) or incubation of oocytes in collected oviduct fluid (Kim et al., 1996), however the polyspermy issue is still unresolved (Yang-Hai et al., 2003). Some factors impact on polyspermy rate during porcine IVF such as high concentration of spermatozoa for performing fertilisation, insufficient IVM oocytes, and suboptimal fertilisation conditions (Hunter 1990; Niwa 2001; Wang et al., 2003). There is the possibility that reducing spermatozoa concentration can decrease the polyspermy frequency, but it can also decrease sperm penetration rate (Abeydeera 1997; Wang 1991; Niwa 2001; Wang 2003; Funahashi 1993; Barbo et al., 2003). In 1992, ICSI was performed to decrease polyspermy incidence (Nakai et al., 2003), although this solution was not very successful because the

procedure led to failures in the oocyte activation, sperm head decondensation, and Zona Pellucida (ZP) breaks (Catt 1995; Flaherty 1995; Hewitson 2000; Li 1999; Shirazi et al., 2009). Another tested approach to decrease polyspermy was zygote centrifugation to assist pronuclei visualisation and selection of monospermic embryos, although the technique is affected by a significant error rate (5-20%) (Gil et al., 2008). The sperm to oocyte ratio is close to unity when the first sperm penetrate, and only increases after the zona reaction has occurred, as the frequency of polysmery is correlated with the number of sperm per oocyte at fertilisation (Hunter et al., 1993; Rath et al., 1992). However, reducing the sperm to oocyte ratio could not overcome polyspermy problem, because decreasing this ratio results in decreasing the sperm penetration rate. It has been found that supplementing IVF media with specific oviduct factors such as oviduct-specific glycoprotein, hyaluronan play an important role in increasing fertilisation rate by decreasing polyspermy frequency (Kouba 2000; Suzuki 2000; Hao et al., 2006). Coy and colleagues (2008) found that exposing oocytes to oviduct fluid makes their ZP resistant to sperm penetration. This resistance happens first through the binding of an oviduct-specific glycoprotein to ZP glycoprotein, then through the stabilisation of the ZP complex by the binding of glycosaminoglycan, such as heparin. Also, some other methods have been developed to decrease the number of sperm reaching the oocyte. These methods are known as biometric microchannel IVF system, straw IVF, modified swim up technique, and microfluidic sperm sorter (Clark 2005; Li 2003; Park 2009; Sano et al., 2010). These approaches can reduce the polyspermy rate, but do not abolish the problem completely. The Funahashi and Romer research group reported in 2004, that brief gamete incubation in the supplemented fertilisation media with caffeine, revealed a reduced rate of polyspermy, however this finding depends on the sperm to oocyte ratio (Funahashi et al., 2004), and it should be optimised according to the impact of boar or storage on sperm fertilisation potential (Gil 2008; Alminnana et al., 2005). However, using a

caffeine supplement may induce spontaneous acrosome reaction which may cause polyspermy (Gil et al., 2008). Other studies have suggested that compounds such as adenosine and fertilisation promoting peptides have the ability to induce fertilisation without significantly increasing the frequency of polyspermy (Funahashi et al., 2000).

1.11.2.2   Oocyte maturation issues

Immature pig oocytes can be retrieved from abattoir material and cultured until maturity, with an oocyte maturation rate of 75%-85% or higher reported in most laboratories (Yuan & Krisher, 2010). Complete oocyte maturation includes the nuclear and cytoplasmic element. Nuclear maturation can be achieved by standard IVM protocol, but cytoplasmic maturation rate is not satisfactory using this method. This can have considered as co factor of high polyspermic rate and low embryo development rate after IVF of IVM oocytes (Gil et al., 2008) (Gil et al., 2010). Development of a suitable medium plays an essential role in improving oocyte maturation and numerous supplements have been examined in maturation medium, for example: calf serum, porcine follicular fluid (pFF), gonadotrophin and epidermal, cysteine, cystamine, glutamine, beta-mercaptoethanol, 9-cis retinoic acid, hormones and growth factor (Abeydeera 2002; Grupen 1995; Day 2000; Aminana et al.,2008). Most of these chemicals are included in common maturation media: NCSU23, NCSU37 and TCM199. pFF suggested as an important factor in protecting oocyte from oxidative stress and its supplementations resulted in the best maturation rate, although its detailed function is not understood well yet (Tatemoto et al., 2004). Moreover, using matured oocytes in a chemically defined media can be resulted in successful piglet production in IVF treatment (Yoshioka 2008; Mito et al., 2009). In addition, important factors which impact on oocyte maturation rate include selecting appropriate oocytes; they are normally aspirated from ovaries of slaughtered animals, as oocytes from different phases are mixed together.

Marchal and colleagues indicated that oocytes which were obtained from large follicles (more than 5mm in diameter) have more potential to develop to embryos than oocytes generated from smaller follicles (less than 3mm in diameter), although controlling follicular size is quite difficult and in pig and cattle follicle size is seasonal. For instance, follicle size was significantly larger during winter, while follicle size considerably small during summer (Marchal et al., 2002; Zeron, 2001; Bertoldo et al., 2011). After oocyte aspiration from appropriate follicle, oocytes are selected for maturation based on their morphology. Oocyte morphology is evaluated according to criteria which includes the number of cumulus cell layers and assessment of the granulation of the cytoplasm, while morphological criteria differ among investigators (Marchal et al., 2002). Kewak and colleagues in 2014, for the first time reported that oocytes generated from large follicles showed reduced IVM time, their maturation time changed from 40-44 hours to 18 hours (Kewak et al., 2014). Reduced IVM time on obtained oocyte from large follicles can result in decreasing oxidative stress level in cumulus-oocyte complex (CCOs, and therefore the reacted oxidative stress (ROS) will be less in matured oocytes from large follicles. It also results in higher oocyte maturation rate, as high level ROS is harmful for oocyte and can damage the cell membrane affecting embryo development and causing early embryo death (You et al., 2010).
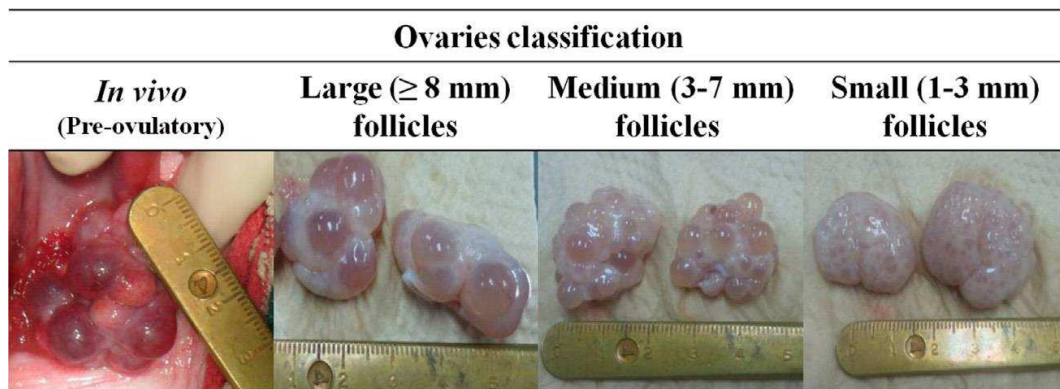
Figure 1.11. Ovary classification based on follicle size. This image taken from (Kewak et al., 2012).

1.12 Boar semen quality and its impact on porcine IVF

Apart from obtaining good quality porcine embryos in order to get higher IVF success rates, semen quality should be evaluated. Pig production in AI and IVF is highly correlated with semen fertilising capacity. Quality of boar semen's one of the most important variables in pig IVF. The effect of boar quality on IVF efficiency has been reported (Gil et al., 2008). Boar semen sub fertility causes economic losses for breeder and also semen quality play an important role in IVF success (Broekhuijse et al., 2011). Boar semen fertility potential varies according to breed line and genetic factors (Wimmers 2005; Lin et al., 2006). Also, boar semen quality can change over a short period of time because of seasonal patterns (Sancho et al., 2004), environmental effects (Murase et al., 2007) and infections (Bussalleu et al., 2011). Semen quality variables include semen concentration, sperm motility, semen morphology, capacitation rate, DNA damage and chromosomal factors. Semen parameters which are important for fertile boar is presented in table 1.7.

Parameter	Requirement
Ejaculate Volume	150-250 ml
Sperm concentration	150×10^6 sperm/ml
Motility	70% progressive motility
Morphology	85% typical forms

Table 1.7. Parameters required for identification of fertile boar semen. This table is based on data from Orsztynowicz et al., 2011.

1.12.1 Techniques to evaluate boar semen quality

Evaluating boar semen quality based on breeding tests is costly and time consuming, therefore, several techniques are used to assess semen quality. The following sections discuss the approaches used to select better quality semen samples.

1.12.1.1   Motility assessment

Sperm motility is one of the important parameters in semen quality evaluation; this technique has been considered as the most commonly tested parameters for artificial insemination (AI) purposes because this test is simple, quick and inexpensive (Sancho et al., 2004). Motility evaluation is performed by determining the proportion of progressive, non-progressive and immotile sperm by microscopy examination. It has been suggested that motility can be an indicator of the intactness of the membrane and functionality (Gadea et al., 2005). Motility has been suggested as a capable seminal parameter because there is considerable correlation between motility and piglets farrowing rate (Gadea, 1998; Gadea, 2004; Sellés et al., 2003). Several studies indicated that the correlation between sperm motility and fertility are conflicting (Tardif 1999; Xu, et al. 1998). It has been reported that assessing

sperm motility by microscope is not very accurate (Foote et al.,2003). An approach to overcome this problem was the development of a computer assisted sperm analyser device (CASA) which resulted in a significant correlation between fertility and motility, although this technique had some limitations, because the accuracy of the CASA device depends on the training of staff, on the calibration, validation, and standardisation of the device

(Holt, 1997; Verstegen, 2002; Broekhuijse et al., 2012).

Other factors can affect boar sperm motility. For example, changing temperature can influence boar sperm motility (Lopez Rodriguez et al,. 2012), although this problem can be solved by incubating sperm at 37C⁰ for 15 or 20 minutes ., Also motility can be increased by adding caffeine as a supplement to fertilisation media It is important to note that there can be a massive variation between different breed of boars and also from the same individual with semen collected at different times (Quintero-Moreno 2004;Ramió 2008;Ramió-Lluch, et al.,2011) .Bacterial contamination has been suggested as another important factor that can affect sperm motility and other kinetic parameters. It was reported that verotoxigenic and enterotoxigenic strains of Escherichia coli significantly reduce the progressive motility of sperm (Bussalleu, et al. 2011).

1.12.1.2   Boar sperm morphology

The morphological features of boar spermatozoa were examined by Retzius 1909 for the first time, but they did could not distinguish boar spermatozoa morphological features in depth. The reason was that the detailed description given by Retzius was based on material collect at autopsy and might not apply to spermatozoa in ejaculated semen. Therefore In this classical research, there was confusion about boar sperm morphological features (McKenzie, 1938; Rao, 1949 Holst, 1949; Lasley et al., 1944). Hancock and colleagues in 1956 continued studying boar sperm morphological features in more detail

compare to previous studies, and the difference was, this group studied sperm

morphological features of ejaculated semen (Hancock et al., 1956). Hancock group (1956)

could examine the boar sperm morphological features, particularly acrosome morphology

in more details. They distinguished outer acrosome and inner acrosome. They described

that outer acrosome is larger which covers the posterior third of the head. The inner

acrosome has a creasentic structure. His group introduced the equatorial segment which

is an area surrounded by the hollow posterior border of the inner acrosome and by the

straight posterior boundary of the outer acrosome. The equatorial segment described as

the area of overlap between the larger outer acrosome and the smaller inner acrosome.

The information was given about the structure of midpiece and tail by Hancock research

group agreed with previous research group results (Hancock et al., 1956). Hancock group

reported that the cytoplasmic droplet is attached to the distal end of the middle piece. It

was found that the acrosome morphological feature is quite depends on boar spermatozoa

storage in vitro. Pursel and colleagues in 1966 studied on the impact of temperature on

boar sperm acrosome morphology. It was reported that sperm acrosome morphology

changed by cold shock at at 0, 5, 10 and 15 C for 10 minutes and this group indicated that

sperm acrosome developed cold shock resistance during 2.5 and 4.5 hours' incubation at

30 C (Pursel et al.,1966). Another study indicated that the osmolarity of the staining

solution and the duration of sperm exposure to the staining solution before smearing or air

fixation suggested as a key element affecting acrosomal morphology. Also, another group

suggested that Sperm acrosomal morphology of extended frozen or thawed semen was

more vulnerable to these factors than was that of fresh semen (Bamba et al., 1973).

Research in boar sperm morphological features did not perform for a long time as traditional

researches achieved reasonable description in terms of boar sperm morphological features

and factors that impact on morphological features. The impact off boar sperm morphological

features on fertility potential became important as the breeds fertilisation potential play an important role on farm industry. Therefore, Bonet and colleagues in 1991 suggested that many boar sperm morphological abnormalities have been associated with infertility (Bonet and Briz et al., 1991). Other studies found no correlation between sperm morphological abnormality and fertility potential (Gadea 1998; Martinez 1986; Xu, 1998; Waberski, 1990; Zeuner et al., 1992). Hirai and colleagues reported correlation between sperm head morphology and fertility, sperm morphology was evaluated by automated sperm analyser (ASA) technique in this research (Hirai et al., 2001). In 2005, the German Umbrella Association for pig production (ZDS-Zentralverband der Deutschen Schweineproduktion) explained that if boar semen exhibits up to 25% morphological abnormal characteristics it can be considered as morphological normal semen (Schulze et al., 2014). Thundathill and colleagues in 2001, found that bull sperm with proximal cytoplasmic droplet abnormalities are not able to bind to the ZP, this group found lower fertility rates, as it has been reported that incidence of proximal cytoplasmic droplet in sperm is associated with sperm immaturity and has an impact on litter size rate as well (Thundathill et al.,2001). However, another study from a different group a few years later reported no correlation between proximal cytoplasmic droplet and bull sperm fertility potential (Quintero-Moreno et al., 2004). Previous studies indicated that there is positive correlation between acrosome morphology and sperm fertility potential (Galli and Bosisio et al., 1988). It has also been demonstrated that only sperm with an intact acrosome initially can bind to the ZP of the pig oocyte (Fazeli et al.,1997). Lovercamp and colleagues suggested that boars with lower fertility and farrowing rates had a significantly lower amount of morphologicaly normal features (Lovercamp et al.,2007). It has been reported that morphology of head and tail considerably impact on sperm motility (Gil et al., 2009). A more recent study confirmed that there is a correlation bettween boar sperm head morphology, retained cytoplasmic droplet and litter size rate

(McPherson et al., 2014). Furthermore, sperm membrane intactness is crucial for proper sperm metabolism and function (Harrison et al., 1996). Numerous authors reported that the membrane structure is not strongly associated with boar sperm fertility potential (Gadea, 1998; Gadea 2004; Tardif et al., 1999), although some other authors achieved inverse outcomes regarding correlation between boar sperm acrosome morphology and fertility potential compared to previous studies. They found that a large number of abnormal acrosomes is correlated with infertility; however no significant correlation between normal acrosome region (NAR) and fertility was indicated (Hammitt, 1989; Sellés, 2003; Perez-Llano, 2001; Flowers et al., 1996).

Therefore, sperm membrane can be considered a marker of sperm vitality and it plays an essential role to maintain sperm function, some handling procedures such as dilution, type of semen extender, storage of sperm in unsuitable temperature (boar semen should be stored at 17℃) can damage the membrane and can result in reduced fertility po tential (Leahy, 2011; Waberski et al., 2011).

1.12.1.3   Techniques used to evaluate boar sperm morphological features

Semen morphology can be assessed using different commercially available stains. Microscopy observation at 1,000x magnification allows distinguish between normal and abnormal spermatozoon (Bonet et al., 2012). Table 1.8 describes the typical forms of boar spermatozoon morphometric parameters, and table 1.9 demonstrates abnormalities.

Parameter	Size (µm)
Head Length	9
Head width	4.7
Flagellum Length	45
Total Length	54.5

Table 1.8. Boar spermatozoon morphometrical parameters (Wysokińska et al., 2009).
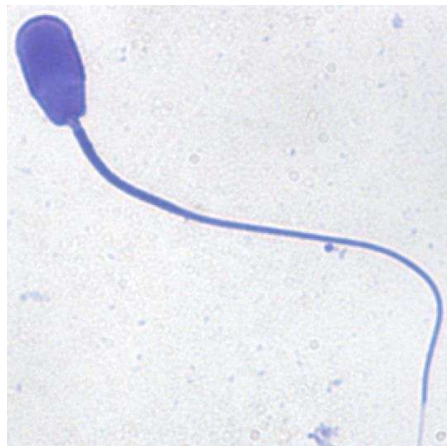
Figure 1.12. Typical morphologically normal boar spermatozoa. As was observed at 1,000X magnification and stained by Sperm Blue®. Different parts of spermatozoa were indicated as: acrosome region (A), occupies nearly 60% of head volume; Midpiece (M) can be differentiated from Tail (T) as it has greater thickness.

Type	Classification
Mature spermatozoon	Normal
Immature spermatozoon	Proximal droplet Distal droplet
Abnormal Head	Head size /shape Isolated head Multiple head
Abnormal Tail	Folded tail Coiled tail Multiple tails
Abnormal acrosome	Absent acrosome Acrosome shape/size Lifted acrosome Detached acrosome
Other	Midpiece abnormality Vacuolisation

Table 1.9. Classification of common boar semen morphologically abnormal features. Taken from (Bonet et al., 2012)

The first evaluation of sperm morphology was determined by monitoring unstained sperm sample under contrast light microscopy. Several staining techniques are available to evaluate sperm morphology in details. These staining techniques are known as: Papanicolaou, Eosin-nigrosine, Trypan Blue, Giemsa, Diff-Quick, and more recently SpermBlue® (Kruger, 1996 Shipley, 1999; Van der Horst et al., 2010). Papanicolaou staining was considered to be a suitable technique to assess sperm morphology for many years according to the world health organisation criteria (WHO) (Organization, 1999;Menkveld et al.,1990) and also this staining can be used for automated sperm morphology analysis (ASMA) (Coetzee et al., 2001). Papanicolaou staining does however have some limitations, as it is very time consuming technique because it includes more than

20 steps and in addition, more than 12 various chemical solutions are involved in performing this staining. The other negative point of using this staining was that as alcohol fixation and xylene dehydration are the main part of the procedure these two steps can cause cell shrinkage (Ross et al., 1953). Clinics and research laboratories required simpler, quicker and safer technique to assess sperm morphology, therefore other staining techniques were introduced by researchers, such as: Diff-Quik, Hemacolor and Giemsa. These staining techniques compared to Papanicolaou staining, are quicker and fewer steps are involved, as they include fixative step followed by staining step with two staining solutions. Diff Quick staining was proposed to be a fast technique as it had only one fixative solution and one staining solution, but this technique had a number of difficulties because it was found that this staining causes sperm swelling and background staining (Kruger, 1988; Organization et al., 1999). A few years later Eosin-nigrosine staining was introduced and was suggested for animal use as well as human. The difficulty with this staining is that it does not discriminate the different components of the sperm (Björndahl, et al. 2003; Van der Horst et al., 200). More recently, sperm blue staining was introduced as an appropriate staining to evaluate sperm morphology much more accuracy than previous techniques with the potential to stain the different components of sperm like the acrosome, head, midpiece and flagellum. This staining technique works very well in both human and animal sperm. The whole procedure takes only 25 minutes and morphology evaluation can be performed accurately in a much shorter period of time compare to previous staining techniques (Van der Horst et al., 2010).

Other techniques to evaluate sperm morphology include techniques the application of fluorescent dyes to stain intact or damaged spermatozoa. These dyes are quantified by counting sperm cells under fluorescent microscope or by a flow cytometer (Ericsson, 1993; Althouse, 1995; Christensen et al., 2004). The most commonly used fluorescent dye in

porcine andrology laboratories is the SYBR14-PI stain that allows discrimination of live and dead or moribund spermatozoa (Garner et al., 1995). Various probes have been developed to assess membrane, acrosome integrity and mitochondrial function De Andrade, et al. 2007). However, to obtain accurate results by applying fluorescent dyes, a large number of sperm cells should be counted under a fluorescent microscope, making it very time consuming. Instead, use of a flow cytometer has been suggested to resolve this problem, as thousands of sperm can be counted under a fluoresent microscope, making it very time consuming. Instead, use of a flow cytometer has been suggested to resolve this problem, as thousands of sperm can be counted in a short period of time (Petrunkina et al., 2010).

1.13 Boar sperm cryopreservation

Exchanging genetic material among breeding populations with liquid stored semen is difficult as a result of the short life-span of liquid stored spermatozoa (Wagner et al., 2000) a workaround could be boar sperm cryopreservation. Semen cryopreservation can also be useful to the pig industry for a number of reasons such as: preserving of superior gene pools, increasing genetic improvement, spreading specific lines across countries and decreasing the need for boar transportation (Almlid, 1995; Johnsonet al., 1998). Sperm cryopreservation has proven quite successful in humans and cattle but not in pigs and it has been suggested that boar sperm might be more sensitive to cryopreservation than other species' (Guthrie et al., 2005). The cryopreservation process is made of three steps: cooling, freezing and thawing all of which can damage the membrane's structure and function (Hammerstedt, 1990; Guthrie et al., 2005). Leboeuf and colleagues reported that the freezing process causes biochemical and functional damages to spermatozoa, and results in decreased sperm motility, viability and fertility potential (Leboeuf et al., 2000). Indeed, boar sperm is very sensitive to the temperature changes occurring during cryopreservation (Holt et al.,

2000) as it is particularly vulnerable to temperatures below 15^OC which can trigger the cold shock process (Gilmore, et al. 1996). This sensitivity to cold shock is correlated to the lipid composition of the boar sperm membrane the main fatty acid components of which are docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) .It was found that plasma membrane damage can be caused by changes in the arrangement of the membrane phospholipids (Medeiros, et al. 2002). During cryopresevation, polyunsaturated fatty acids (PFAs) decrease considerably because of lipid peroxidation, therefore to decrease the formation of reactive oxygen species and protect the plasma membrane's function, antioxidants and fatty acids are commonly added to the semen in preparation to cryopreservation. Pursel and colleagues in 1972 suggested that boar spermatozoa can acquire cold shock resistance by keeping them at room temperature in seminal plasma for 1-5 hours (Pursel et al., 1975) .In 1988, it was found that keeping boar sperm at $15\,^O$C for more than three hours before cryopreservation can result in improved sperm motility and fertility potential (Almlid, 1988; Eriksson et al., 2002). When the freezing stage of the cryopreservation procedure is performed, the temperature decreases from $-15\,^O$C to $-60\,^O$C, which causes sperm damage because of the formation of intracellular ice and cellular dehydration. The spermatozoa will lose water rapidly to prevent intracellular ice formation, however this will lead to both cell and plasma membrane dehydration (Mazur, 1970; Parks et al., 1992). Intracellular ice formation and dehydration are considered as the main factors that impact on boar sperm fertility potential after cryopreservation and may lead to sub fertility (Roca, 2004 Roca et al., 2005).

1.14 Sperm capacitation

Natural fertilisation can only occur when the sperm acquires the ability to penetrate the ZP and after its full maturation. During this process, the acrosome goes through the activation process and acquires the ability to penetrate the ZP. This biological change is known as capacitation (Chang et al., 1958). In vivo, capacitation occurs in the female reproductive tract while in vitro it is stimulated by the incubation of sperm and oocytes in an appropriate capacitation medium which needs to contain bicarbonate, calcium and serum albumin (Yanagimachi et al.,1994).Sperm capacitation includes several steps such as: reorganisation of the membrane proteins, regulation of membrane phospholipids, reduction of membrane cholesterol levels, changes in the sperm motility and ability to go through the acrosome reaction (Rathi et al., 2001).

Calcium concentration, protein phosphorylation, acrosome and membrane reorganisation and can be influenced by the changes in the uterine and oviduct fluid resulting from the different stages of the oestrous cycle (Hunter, 1974; Brown et al., 1973). The average time for the completion of capacitation in boar spermatozoa is two hours in vivo (De Lamirande, et al. 1997; Harrison 1996; Yanagimachi et al.,1994). In vivo boar spermatozoa are deposited in the female genitalia well a head of ovulation, gathered in the uterotubal junction (UTJ), where a functional sperm reservoir (SR) is established. This phenomena ensure that an appropriate number of viable, potentialy fertile spermatozoa are available to fertilise the ovulated spermatozoa at the upper amppularly junction(AIJ) (Rodriguez-Martinez et al, 2007). According to in vitro sudies, it has been suggested that capacitation occure in the SR but it is not yet clear how capacitation or sperm release are regulated by the porcine SR (Hunter et al, 1998). However, most studies on capacitation have been done in vitro. As this step is quite essential for fertilisation, and a better understanding of sperm capacitation could lead to better fertilisation outcomes in vitro. Sperm capacitation in vitro can be assessed by

several techniques. Fluorescently labelled lectin has been employed to flag the acrosome reaction (AR) as lectin it binds specifically to the acrosome content (Cross, et al. 1986). Although lectin staining is supposedly relaiable and simple, monitoring the degree of capacitation proved challanging with this staining because of the difficulty in controlling the exposure interval to solubilised ZP and the time required for the spermatozoa to be permeabilised and fixed. Therefore, to evaluate acrosome reaction, another technique was adopted which was much more reliable and quick: chlortetracycline (CTC) staining (Saling et al., 1979). CTC staining assesses the distribution of fluorescence on the sperm head to identify capacitated spermatozoa. This staining technique first was developed for mouse spermatozoa and then applied gradually to human and boar spermatozoa (Endo, 1988; Fraser, 1990; Saling, 1979; Ward, 1984; DasGupta, 1993; Lee, 1992; Varner, 1987; Barboni et al., 1995).

Several factors affect boar sperm capacitation. For instance, the capacitation rate is affected by the presence of progesterone which stimulates boar sperm capacitation and can result in higher fertility potential (Barboni, et al. 1995). The capacitation culture media used can also be considered as another important facttor which influences the capacitation rate under IVF conditions. Dapino and colleagues (2005) introduced heparin as an effective supplement for capacitation and suggested that adding 10mM heparin in the capacitation media can induced the acrosome reaction and results in increased capacitation rates (Dapino et al., 2005). Also, the same research group reported that calcium and bicarbonate are required components for capacitation but their combination with heparin stimulates capacitation even further (Dapino et al., 2006).

Caffeine and adenosine were also introduced as an effective supplement to improve the sperm motility and the capacitation rate in porcine IVF systems (Harrison, 1993; Funahashi et al., 2000). Caffeine stimulates an increase in intracellular levels of 3', 5'-cyclic adenosine

monophosphate. However, caffeine also stimulates spontaneous acrosome reaction which in turn might cause polyspermy in porcine IVF, therefore carefully regulating the amount of supplemented caffeine is very important (Funahashi et al., 2001).

Song and colleagues in 2008 reported the possible role of chondroitin sulphate in the induction of capacitation in boar spermatozoa following the observation that porcine follicular fluid includes a variety of glycosaminoglycan, mainly chondroitin sulphate, generated by the granulosa cells (Song et al., 2008). De Oliveira and colleagues (2011) indicated that chondroitin sulphate increases fertilisation rates but does not increase blastulation rates and therefore cautioned that chondroitin sulphate might inhibit embryo development, that is why it is not very common to use it in fertilisation media (de Oliveira et al., 2011).

Approximately 15% of couples suffer from infertility and a male factor is likely contributing in half of these cases (Oehninger et al., 2001). Tipically, sperm motility, morphology and capaciation assessment were used in most andrology laboratories to determine diagnose a male factor. Although an accurate diagnosis of male infertility can be achieved by these techniques (Centola and Ginsburg et al., 2004), they also have some limitaions. For instance, when a sperm quality parameter falls short in a particular male, that individual's fertility potential can somettimes be compensated by regulating the quantity of sperm used in IVF. Thus more information is required at cellular and molecular level to discriminate fertile and subfertile males in order to recognise more factors that are possibily correlated with uncompensable charachteristics (Salisbury, 1961; Pace et al.,1981; Saacke et al.,2000; Sackee et al.,1994).

1.15 Mammal sperm chromatin structure

Recently, research has focused on abnormalities in the male genome that can impact on fertility and are caused by DNA damage and abnormal chromatin packaging (Lopes, 1998; Sakkas et al., 1999). Chromatin organisation and packaging differ between gametes and somatic cells (Helen et al. 2005). In particular, sperm chromatin packaging is characterised by the presence of protamine in place of histones, which are observed in other cell types (Dadoune et al., 1995). Histones are proteins that specifically bind DNA and play an essential role to condense the DNA into a smaller organised structure called chromatin. Histones are rich in positively charged amino acids, particularly arginine, and in cysteine residues form disulphide cross links which ensures high stability of the chromatin structure (Bianchi et al. 1996). Thanks to protamines, the DNA in mammalian sperm is nearly six times more condensed than in any other cell type (Ward and Coffey et al., 1991). The chromatin content of sperm contributes to 50% of a zygote's genomic material and pre-implantation development (Tavalaee et al., 2014). Thus, evaluation of sperm chromatin integrity may assist to predict sperm fertility potential . During spermatogenesis, round spermatids go through remarkable changes, including loss of most (not all) of the cytoplasm and the development of a motile tail. During these stages, a highly condensed sperm nucleus is produced with reduced replicative, translational and DNA repair activities. This process encompasses various complicated steps during which histone and non-histones chromatin proteines are substitued with one or more protamine types in the process known as condensation (Loir et al.,1978). In rat, mouse and sheep this replacement is assisted by a set of proteins known as transition proteins. Histone substitution with protamines causes a very significant change of the chromatin structure which is important for nuclear organisation (Fuentes et al.,2000). Protamines are half the size of histones. It is known that arginine is the most common aminoacid in histones which palys a crucial role in creating

very strong DNA binding. Instead, cysteine is the most common aminoacid in protamines and that has an essential role during the final stages of nuclear maturation as cysteine contributes to chromatin compaction by establishing multiple inter- and intra-protamine disulphide cross-links (Loir et al., 1984).

All these changes give mammalian sperm a unique structure providing it with the most condensed eukaryotic DNA (Ward et al., 1984). The condensation procedure plays an important role in appropriate sperm function, and the decondensation process is important as well. When the sperm nucleus is delivered into the ooplasm before fertilisation, the condensed sperm nucleus should go through decondensation immediately in order to release the DNA for the formation of the paternal pronucleus. Therefore, decondensation is a crucial step for the transcription of the paternal genome, and is an important part of the fertilisation process. Decondensation is known as the chromosomal remodelling step and it happens before the sperm penetrates the ZP. Two steps are involved in the chromosomal remodelling: first of all, reduction of the disulphide bonds and secondly replacement of protamine by histones (Yanagimachi et al., 1994). The mechanism by which protamine are replaced by histones is still unclear. Protamine removal initiates when the sperm begins to penetrate the ZP (Perreault 1992), while it is very unknown when the decondensation process exactly happens (Nonchev and Tsanev et al., 1990). It has been suggested that compacted chromatin is less vulnerable to DNA damage as comapred to less compacted chromatin (Aoki et al.,2005). It has been found that protamines has a possible role in silencing the paternal genome and on imprinting (Aoki and Carrell et al., 2003). Previous research suggested that abnormal histone replacement can affected male fertility potentials (Ward et al., 2010).
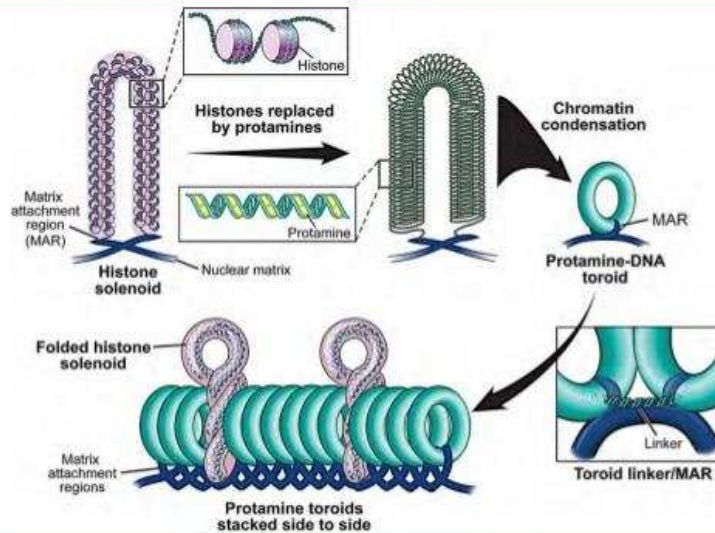
Figure 1.13. Protamination procedure in sperm. During spermatogenesis, histones are replaced by protamines to achieve high sperm nucleus condensation. The image was taken from http://www.slideshare.net/sandroesteves/sperm-dna-fragmentation-in-male-infertility.

In addition, zinc (Zn) is considered important for sperm chromatin integrity. During spermatogenesis, at the stage when the sperm nucleus compaction has started, Zn deficiency can result in the lack of elongated spermatozoa and severe Zn deficiency blocks the formation of spermatozoa (Barney et al., 1968). The human sperm chromatin contains one Zn ion for each turn of DNA and for each protamine molecule. It can be concluded that zinc is required for DNA-protamine as illustrated in figure 1.16 (Bench 2000; Kjellberg et al., 1993).
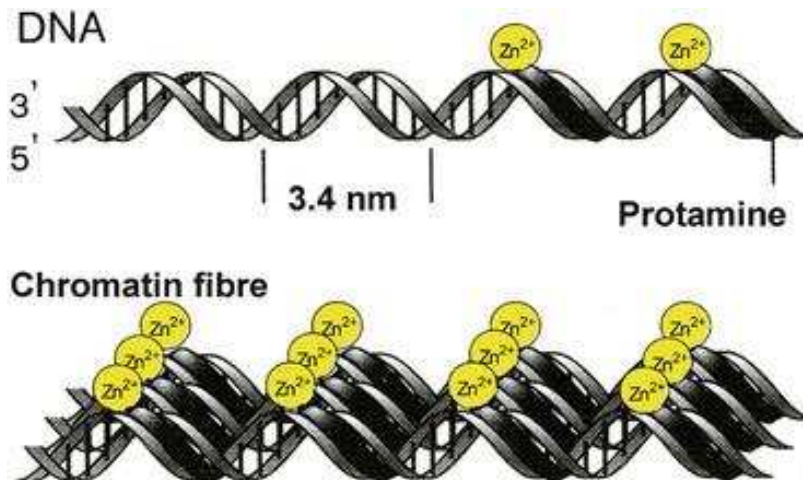
Figure 1.14. Sperm chromatin packaging. The DNA double helix in sperm chromatin and its correlation with protamines is shown, demonstrating the probable role of Zn in connection with protamines. There is one Zn per protamine molecule for every 10 pairs of nucleotides. The image was taken from (Björndahl et al., 2014).

1.15.1 Correlation between sperm chromatin packaging and fertility potential

It has been suggested that sperm chromatin packaging abnormalities can occur because of defective protamination (Balhorn 1988; Yebra et al., 1993) and existence of DNA breaks. Many studies highlighted a correlation between decreasing fertility potential and decreasing normal sperm chromatin packaging (Hoshi 1996; Bianchi 1996; Sakkas 1996; sun 1997; Lopes et al., 1998).

In the breeding industry, the assessment of boar sperm chromatin structure is one of techniques that can be used to estimate a boar's fertility potential, therefore the impact of protamination on the epigenetics of mature sperm cell attracted much attention (Carrel 2010; Jenkins et al., 2011). That is why different staining techniques for assessing sperm chromatin packaging and measuring the quantity of protamines are currently under investigation (Iranpour 2000; Kazerooni et al., 2009). It has been reported that protamine deficiency and sperm DNA damage can cause male infertility or sub fertility in both natural and assisted conception (Tarrozi et al., 2009). Cooper and colleagues reported that sub

fertility in males could be correlated with incomplete replacement of histones by protamines (Cooper et al., 2009). Reduction in protamine expression is quite common in infertile men, while it is uncommon in males with proven fertility (Carrell et al., 2006). It has been suggested that men with protamine deficiency display low sperm count, poor sperm motlity, poor morphology and low sperm penetration rates (Aoki 2003; Carell 2001; Aoki et al., 2005). It is not clear whether there is a correlation between protamine deficiency and defects of spermatogenesis, and no protamine level thresholds has been established yet to determine impact on semen quality. However, determining sperm protamine levels may assist in selecting better sperm for use in ICSI and other assisted reproduction techniques (Allen and Reardon et al., 2005).

1.15.2 Techniques for the determination of sperm chromatin packaging

Protamines can be isolated and sperm protamine content can be quantified by electrophoresis (de Yebra, 1998; Aoki, 2005; Balhorn et al., 1988), although this technique is time consuming for routine diagnosis (McKay1986; Yelick et al. 1987). Chromomycin A_3 (CMA_3) staining has been introduced as an appropriate technique for protamine detection and can applied to human, mouse, bull and boar sperm (Bianchi, 1996; Zubkova et al., 2005).

1.15.2.1   Chromomycin A3 (CMA3)

CMA_3 is an antibiotic generated by Streptomyces griseus which was known previously in nuclear medicine as Toyomycin. CMA3 is an anthraquinone antibiotic with florescent properties which inhibits RNA synthesis. It has been reported that CMA3 has antibacterial, antifungal and anti-neoplastic abilities (Chakrabarti et al. 2000). CMA3 binds as a Mg^{2+} co-ordinated dimer at the minor groove of GC- rich DNA regions and stimulates a conformational disturbance in the DNA helix (Chakrabarti 2000, Berman et al., 1985). This makes the CMA3 a strong competitor to protamines (Nijs et al., 2009). A positive CMA3

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staining indicates protamine deficiency while a negative CMA_3 result suggests no protamine deficiency (Iranpour et al., 2000). CMA3 has been used to evaluate protamine deficiency in both human and boar sperm and it is a very quick technique to determine sub fertility in males (Simoes et al., 2009).

1.16 Sperm aneuploidy

Spermatozoa are the end product of spermatogenesis; spermatocytes go through two meiotic divisions during spermatogenesis before haploid spermatids are generated, then spermatids develop into mature spermatozoa during a series of molecular and cellular events (De Jonge et al., 2000). In humans, a mature spermatozoon has 22 autosomes and one sex chromosome while boar spermatozoa contain 18 autosomes and one sex chromosome. Incorrect chromosome segregation during the first or second meiotic divisions results in chromosomally abnormal spermatozoa with an improper chromosome number (aneuploidy) or spermatozoa with additional or deleted chromosomal material (unbalanced spermatozoa). Fertilisation with chromosomally abnormal spermatozoa can result in chromosomally abnormal embryos (Carrell et al., 2006) and it has been suggested that chromosomal abnormalities in embryos are the main cause of pregnancy loss and foetal malformations (Hassold et al., 2001) .The proportion of paternally inherited aneuploidy is small compared to the maternal factor (Pacchierotti et al., 2007).

1.16.1 Aneuploidy in boar spermatozoa

High rates of chromosomal abnormalities in boar semen may lead to decreased fertility but not necessarily to decreased fertilising potential (Orsztynowicz et al., 2011). Recently, new attempts have been made to optimise fluorescence in situ hybridisation (FISH) to evaluate semen for chromosomal abnormality levels. Currently, only a limited number of studies have been performed in order to estimate aneuploidy in boar sperm. When this testing has been

performed on chromosome 1, 10 and Y no significant difference was observed among breeds in terms of aneuploidy (Massip 2009; Massip 2010; Pinton 2009; Bonnet-Garnier et al., 2009). FISH has been reported as an appropriate tool to identify chromosome abnormalities, particularly aneuploidy, so this technique could be used to asses sperm fertility by complementing morphological and functional assessments with a genomic screening. In order to use FISH to estimate aneuploidy rates in sperm, the first step needed is decondensing the tightly packed sperm nucleus (E Downie et al., 1997).

1.16.1.1   Evaluating sperm aneuploidy

Two techniques are available to determine aneuploidy in sperm cells. One of these techniques is the analysis of the sperm karyotype after the in vitro fusion of a hamster egg and a human sperm (Rudak et al., 1978), while the other technique is multicolour FISH, which allows screening sperm chromosomes in interphase nuclei (Holmes & Martin et al., 1993). Both these techniques have restrictions, as only a small number of cells can be examined with the hamster technique and a limited number of chromosomes can be screened by FISH. Another difficulty with FISH is that it tends to detect artificially high levels of nulliosomy due to artefactual loss of chromosomes during slide preparation. Despite its limitations, FISH is still the most commonly used technique as it can be performed quickly and a large cohort of spermatozoa can be analysed in one experiment therefore increasing the accuracy of outcomes.

It has been found that infertile men with normal karyotypes have an increased risk of sperm aneuploidy (Palermo et al., 1992). The correlation between sperm concentration and sperm aneuploidy rates have been studied and a strong and inverse correlation has been found between sperm concentration and sex chromosome aneuploidy (Castro 2009; McAuliffe et al., 2012b). Sarrate and colleagues reported a threefold increase of chromosome 21 disomy in patients with oligozoospermia (low sperm count), and a two to threefold increase in sex

chromosome disomy in these patients compared with the control group (Sarrate 2010; Mougou-Zerelli 2011; Durak Aras et al., 2012). The correlation between chromosome abnormality and sperm motility is quite controversial. The majority of research studies indicate no significant correlation between sperm motility and disomy incidence (Sarrate 2010; Mougou-Zerelli et al., 2011). However, other groups reported an increase in aneuploidy rates in patients with asthenoteratozoospermia (poor sperm motility and morphology) but normal sperm concentration (Hristova 2002; Templado 2002; Collodel et al., 2007a). Moreover, Zeyneloglu and colleagues (2000) suggested that immotile spermatozoa with morphologically normal features do not correlate with increased aneuploidy incidence, while a high incidence of aneuploidy (mainly diploidy and sex chromosomal aneuploidy) was observed among patients with dysplasia of the fibrous sheath (DFS), a flagellar pathology causing severe sperm immotility (chemes 1987, Torikata 1991; Rawe et al., 2001).

The correlation between sperm morphology and aneuploidy is still not entirely obvious. Several studies demonstrated a higher risk of aneuploidy among patients with teratozoospermy (presence of high levels of sperm with abnormal morphology) (Kahraman 2004; Dubey et al., 2008). Furthermore, several research studies investigated whether sperm morphology is a viable indicator for the selection of chromosomally normal sperm (Gole 2001; Templado 2002; Brahem 2011a, 2012; Mougou-Zerelli et al., 2011). Diploidy, triploid and tetraploidy rates were found to be significantly increased in patients with macrocephalic multiflagellated sperm syndrome compared to control samples (Perrin 2008; Brahem et al., 2011a).

In comparison with humans, data on the frequency of chromosomal abnormalities in farm animals are much more limited because there is no precise monitoring of embryos during development and samples from miscarriages and abnormal animals are only occasionally

sent to perform further cytogenetic tests. However, numerical chromosomal abnormalities such as trisomy of particular chromosomes, monosomy of chromosome X, polyploidy and also structural chromosome abnormalities like reciprocal and Robertsonian translocations, inversions or insertions have all been found in farm animals (King et al., 2008). The results obtained in farm animals in terms of types of chromosomal abnormalities are quite similar to humans', while the frequency of chromosomal abnormalities is variable among gametes or embryos of different species (Zuccotti 1998; King et al., 2008).

1.16.2 Nuclear organisation (chromosome positioning)

The hypothesis of non-random nuclear organisation was suggested by Carl Rabl and Theodor Boveri for the first time at the end of the 19th. It is now well understood that chromosomes occupy discrete territories (Cremer 1993; Lichter 1988; Manuelidis and Borden et al., 1988), the exact chromosome distribution depends on the stage of the cell cycle and on the cell type (Funabiki 1993; Mayer et al., 2005). Nuclear organisation and its functional roles are highly conserved in eukaryotes (Neusser 2007; Tsend-Ayush et al., 2009). It has been suggested that chromosome territories (CTs) have a radial organisation and the most gene-rich chromosomes are located in the centre of the nucleus while gene-poor chromosomes are in the more peripheral regions of the nucleus (Boyle 2001, Cremer et al., 2003). Furthermore, the radial organisation plays a protective role for the genome. Chromatin, which is located in the periphery of the nucleus, can be considered as a shield that absorbs mutagens as they enter the nucleus and protects the central part of the nucleus from damage (Hsu 1975; Tanabe et al., 2002). Several studies have indicated that nuclear organisation plays a crucial role in ensuring that suitable regions of the genome are positioned by replication and transcription cues. Moreover, it has been reported that nuclear organisation plays an essential role in maintaining the correct orientation of chromosomes

within the nucleus, which is important during cell division, so that appropriate nuclear organisation can assist in regulating the normal function of genome (Cremer et al., 2001). Alterations in nuclear organisation can result in pathologies such as: cancer (Kurodo et al., 2004), laminopathies (Mewborn et al., 2010) and X-linked EmeryDreifuss muscular dystrophy (Boyle et al., 2001). A number of studies reported the dynamic plasticity of chromatin and suggested that while interphase chromosomes clearly occupy restricted nuclear domains, the folding of chromatin within these domains has substantial flexibility (Shopland et al., 2006). This plasticity of chromatin is adequate to form the intra and inter chromosomal interaction that play an essential role in shaping the nuclear prospect (Fraser 2007; Schoenfelder 2010; Groudine et al., 2011).

1.16.3 Sperm nuclear organisation

It has been reported that CT positioning in human sperm cells is non-random (Gurevitch 2001; Hazzouri 2000; Sbracia 2002; Tilgen et al., 2001). CT localisation can be performed by using chromosome (usually centromere) specific probes or using whole chromosome paints (Hazzouri et al. 2000; Zalenskaya and Zalensky 2004; (Mudrak et al. 2005; Zalenskaya and Zalensky 2004; Manvelyan et al. 2008). Some differences in CT positioning has been observed between studies, however this is likely to be due to variation in decondensation procedure used. As sperm nuclei in many mammals have a polarised shape with two poles: apical (acrosome) and basal (tail attachment), it is possible to assess the longitudinal positioning of CTs. This has been done for human (Manvelyan 2008; Mudrak 2005; Zalenskaya and Zalensky et al., 2004) and boar spermatozoa (Foster et al., 2005). Furthermore, the radial positioning of CTs has been observed. Results showed an association between the radial positioning and chromosome size in human sperm, however no correlation was observed between CT size and distribution in boar sperm (Foster et al., 2005). Overall however, most studies agree that mammalian sperm demonstrate a

chromocentric model of nuclear organisation, with centromeres localised deep in the nuclear interior and telomeres clustered at the nuclear periphery. This is thought to be highly important during gametogenesis, fertilisation and embryo development (Zalenskaya e al., 2004).

1.16.3.1   Telomere distribution during spermatogenesis

During spermatogenesis, telomeres lead the 'bouquet' formation of chromosomes during the leptotene stage of prophase I. In mice, rats and humans interior telomeres move to peripheral positions following preleptotene (Prophase I stage), then at the end of preleptotene, telomeres become completely localised at the nuclear envelope. From leptotene to zygote stage, peripheral telomeres develop tight clusters as chromosomes adopt the bouquet formation. By mid of zygotene telomeres of some cells stay clustered, although at this stage telomere begin to diffuse. In pachytene telomeres remain fully dispersed at the nuclear periphery (Meyer-Ficca 1998; Scherthan et al., 1996). Interestingly in cattle telomeres stay clustered in the bouquet formation until late zygotene (Pfeifer et al., 2001).

Based on mouse studies, SUN1 (a specific protein expressed during meiosis) has been shown to be an important protein in attaching telomere clusters to the nuclear periphery and in ensuring homologous chromosome alignment, synapsis and recombination. Ding and colleagues reported that the deletion of SUN1 result in disruption of bouquet development during meiosis and can cause infertility in males and females (Ding et al., 2007). It has been suggested that during maturation, telomere distribution in the sperm appears to alter from peripheral in spermatocytes to more central clustering around the nucleolus in round spermatids, then after the elongating spermatid stage telomeres redistribute back to the nuclear periphery (Tanemura et al., 2005) .This is thought to play a functional role in

anchoring chromosome territories in place, to provide replication and transcriptional cues during fertilisation and embryogenesis (Zalenskaya et al., 2004) .

1.17 Thesis aims

The specific aims of this thesis were as follows:

1. To produce a working classification system for boar sperm morphology and to test the hypothesis that there are differences in sperm morphological features between fertile and sub fertile boar, and also to investigate the impact of sperm freezing technique on sperm morphology.

2. To investigate that whether sperm concentration impacts on fertility rate in porcine IVF procedure, and also to determine the capacitation time for both fertile and sub fertile groups. To investigate the impact of adenosine and caffeine supplements in capacitation rate and also find out wither there is correlation between fertilisation rate and capacitation proportion in order to introduce capacitation as an appropriate predictor for fertilisation. To test the hypothesis that there is correlation between capacitation rate and sperm morphology.

3. To test the hypothesis if chromatin packaging is different in both fertile and sub fertile groups. To test the hypothesis that decondensation process is different in fertile and sub fertile groups. To estimation sperm aneuploidy for autosome chromosomes and also to study the telomeres distribution in both fertile and sub fertile group and test the hypothesis that the telomeres distribution is non-random.

4. To test the hypothesis that the embryo biopsy impact on embryo morphology and also determine the best biopsy stage (cleavage stage or blastocyst stage). To investigate whether advanced maternal age impact on biopsy outcomes. To predict blastocyst quality and pregnancy rate based on time-lapse parameters.

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2   Materials and methods

2.1   Materials

All pig semen samples for the methods described in this section were received from JSR

Genetics Ltd (Deified, UK). Porcine fertile and sub fertile samples were obtained from four

different breeds: Hampshire, Large white, Landrace and Pietrain. Samples with a motility

score of > 60% and normal morphology score of > 50% were classified as fertile and those

with motility of < 60% and morphology of < 50% were classified as sub fertile. Abattoir

derived porcine ovaries were also provided by JSR Genetics Ltd (Deified, UK).

For studying the impact of biopsy on human embryo morphology (see section 3.1.1),

samples were obtained from the Assisted Conception Unit in the Reproductive Centre at

Guy's and St Thomas hospital (London, UK). Data for this research was obtained

retrospectively from 110 couples that undertook intra cytoplasmic sperm injection (ICSI)

treatment from August 2012 to August 2014. Biopsy on day three and day five was

performed for patients undergoing preimplantation genetic diagnosis. ICSI and biopsy

procedures were performed by trained embryologists and were all incubated in an

Embryoscope time-lapse device (Unisense Fertilitech, Denmark) and subsequent

annotation was performed using the Embryo viewer software (Unisons Fertilitech,

Denmark). All protocols were performed under the human fertilisation and embryology

authority (HFEA) licence 0700/L700-18-c awarded to the Reproductive Centre at Guy's and

St Thomas' hospital. Use of this pre-existing data for research purposes was approved by

the Research Ethics Committee of the University of Kent.

2.2   Methods

2.2.1   Sperm sample preparation

Boar semen samples were collected by the gloved hand method as was described by King and Macpherson (King et al., 1973) at a JSR Genetics farm. TRIXcell PLUS® solution (IVM technologies, L'Aigle France) was heated to 20°C and used to extend the semen (2:1 extender to semen ratio). Samples were delivered to our laboratory at the University of Kent and kept at 17°C until use either the same or the next day.

2.2.2   Sperm concentration and motility assessment

Boar semen concentration analysis was performed using a Makler chamber (Sefi medical instruments, Haifa, Israel). The chamber depth is 10µm and the coverslip bears an impressed counting grid divided into 100 0.1 mm^2 squares. When a drop of semen sample was placed on the chamber, the number of motile spermatozoa heads which swim completely or partially in 10 squares is equal to the sperm concentration in millions per ml. The Makler chamber is indicated in Figure 2.2. Semen samples were gently mixed to avoid imprecision caused by spontaneous sedimentation and a 10 µl drop was placed on the centre of the Makler chamber with the coverslip placed over the top. Sperm counting was performed under 200 x magnification using a Zeiss Primo Star optic microscope. The protocol for his experiment was adopted from (Xu et al., 1995).
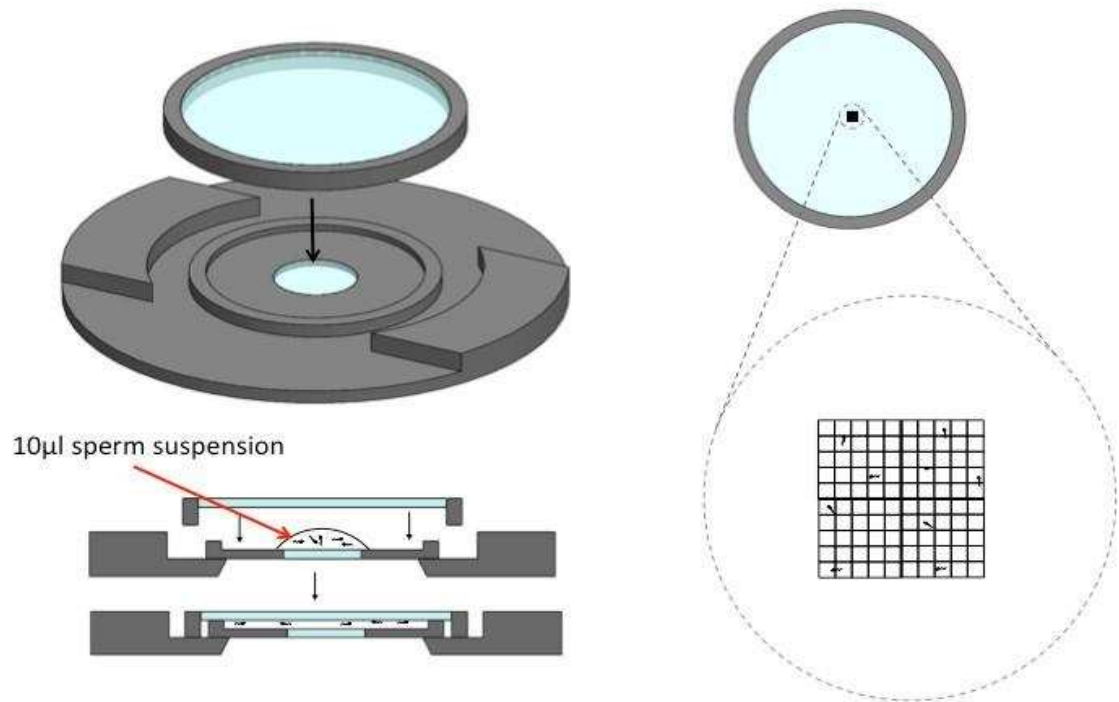
Figure 2.1: The Makler chamber and the process of spermatozoa counting in order to calculate correct spermatozoa concentration. Each of the 100 small squares has surface of 0.1 mm^2. Spermatozoa concentration is evaluated by counting sperm cells in 10 random squares; sperm heads outside of the square were ignored total number of spermatozoa was multiplied by 10^6 to obtain the required sperm concentration of one million per ml for fertilisation protocols.

2.2.3 Sperm Motility evaluation

Motility evaluation was performed by adding 10µl of semen onto slide. A total of 250 spermatozoa were counted on each slide and were classified into three groups; progressive, non-progressive and immotile. This motility classification was established according to World Health Organisation (WHO) procedures. Table 2.2 indicates these criteria for sperm motility assessment. This experiment procedure was adopted from (Sancho et al., 2004)

Motility	Description of motility
Progressive	**Fast and straight movement of sperm with full tail flexion**
Non-progressive	**Limited movement without significant progression**
Immotile	**No movement at all, spermatozoa presumed dead**

Table 2.1: Classification for sperm motility evaluation. 250 spermatozoa were counted for each slide. The percentage of total motility was based on the formula: ((progressive+ non-progressive)/250) x 100

2.2.4 Boar sperm morphology evaluation

To produce a working classification system for boar sperm morphology, sperm blue staining was performed. Morphology assessment was undertaken for 4 breeds for both high quality and poor quality semen groups (see table 2.3 for breed information). 10 µl of a fresh semen sample was smeared on a standard microscopy slide and left to air dry. Slides were placed in Sperm Blue® fix solution for 10 minutes (Microptic, Barcelona, Spain) and allowed to run off the slide onto absorbing paper. Following this, the slide was immersed in Sperm Blue® stain for 20 minutes. Excess stain was removed by dipping into distilled water for 3 seconds;

it was important not to leave slides in distilled water for longer than this to avoid loss of sperm cells. Slides were left to air dry at an angle, allowing the excess stain to run off. Finally, slides were mounted with DPX mounting medium under a 24x50 mm coverslip. For each breed four replicates were prepared, and from each slide at least 250 spermatozoa were examined; as a result, 1,000 spermatozoa were assessed for each boar. Spermatozoa were categorised as either good or poor quality according to their motility and morphological features. Poor morphological features of semen samples were divided into several sub-groups (summarised in Table 2.2). Spermatozoa with multiple abnormalities were scored according to the one with the most severe grade. Observation was performed under 1,000X magnification with standard immersion oil under a Leica DMRB microscope equipped with a Leica DC300 camera and its dedicated software.

Absence of acrosome cap
Lifted acrosome
Detached acrosome
No acrosome at all or acrosome area occupied more or less than 75% of sperm head
Coiled or folded tail
2-tails
2-heads
Cytoplasmic droplet (proximal and distal)
Abnormal midpiece
Vacuolated acrosome
Vacuolated head

Table 2.2: Boar semen morphological abnormal categories assessed in this thesis.

2.2.5 Impact of freezing on boar sperm morphology

In order to assess the impact of freezing on sperm morphology, samples from two white Duroc pigs were obtained and analysed both before and after freezing. The freezing procedure was performed at JSR Genetics Ltd (Driffield, UK). Morphology was assessed by sperm blue staining as mentioned in section 2.2.4. Four slides per animal were prepared and 250 sperm cells per slide were evaluated.

2.2.5.1 Semen freezing and thawing procedure

Shortly after semen collection, the semen was diluted (1:1 v/v) with extender I (Modena^{TM}, swine Genetics International, Ltd., Iowa, USA). The diluted samples were transferred to 50ml falcon tubes, and equilibrated at 15ºC for 120 minutes, followed by centrifugation at

800xg for 10 minutes. After this, the supernatant was discarded and the sperm pellet was re-suspended with extender II (80ml of 11% lactose solution and 20ml egg yolk) to a final concentration of 3.5×10^9 spermatozoa/ml and cooled at $5C^o$ for 90 minutes. Subsequently, extender III (89.5% of extender II with 9% of glycerol and 1.5% Equex-STM®) was added. The final concentration was approximately 3×10^9 spermatozoa/ml contained in 3% glycerol which was loaded into to 0.5ml straws (Bio-Vet, Z.I. Le Berdoulet, France). The straws were sealed with PVC powder before placing in an expandable polystyrene box where the straws were in contact with nitrogen vapour (3cm above the liquid nitrogen level) for 20 minutes. The straws were sunk in to liquid nitrogen at -196^oC. Cryopreserved semen samples were kept in liquid nitrogen for 6 months.

For assessing sperm morphology following freezing, straws were thawed by immersing them in water for 12 seconds. Immediately after thawing, the sperm was diluted (1:10) with PBS and after loading slides, and sperm blue staining was used to assess morphological features of semen samples as mentioned previously in section 2.2.4. This experiment was performed at JSR Genetics Ltd (Driffield, UK) based on their developed protocol.

2.2.5.2 Statistical analysis

To statistically analyse the impact of freezing on boar sperm morphology, a chi-squared test was used. Results were considered significant for $p \leq 0.05$.

2.2.6 Optimising boar sperm capacitation medium in vitro

Fresh semen samples that had been stored for 1-4 days in a commercial extender TRIXcell PLUS® solution (IVM technologies, L'Aigle, France) were used for these experiments. Sperm wash (10mM NaCl, 10mM TRIS pH 7.0, 0.58g NaCl/1.21g Tris per 1L) and pre-incubation medium (ingredients shown in table 2.5) were prepared with osmolarity of 0.260-0.280 Ocm/L and left in the incubator ($37°C$, 5% CO_2, 20% O_2) overnight with loose lids in order to adjust pH. The pH of both medium was checked on the following day, and their pH

was adjusted to 7.4. Caffeine and adenosine was added to 10 ml of pre-incubation medium (2mM caffeine or adenosine per 10 ml capacitation medium) in order to compare capacitation rate. 5 ml of sperm wash media was added to 2ml of fresh semen and centrifuged at 800 x g for 5 minutes. The supernatant was removed leaving the pellet in a residual 2ml of solution for resuspension. Another 5ml of sperm wash medium was added to the sample and the previous step was repeated. Then semen concentration was checked using a Makler chamber as described in section 2.2.1. Following this, semen samples were added to the three-different medium such as capacitation, capacitation caffeine and capacitation adenosine and were incubated at 38°C in the incubator for 240 minutes. Samples were collected every 30 minutes and 50µl sample was added to 50µl of 750µM chlortetracycline hydrochloride (CTC) (Sigma, UK) solution (0.2mg CTC, 20µM Tris, 130µM NaCl, 5µM cycteine, pH: 7.8). CTC solution was made fresh for each new experiment and kept in the fridge until use, in the dark as it is very light sensitive. After 20 seconds, the reaction was stopped by the addition of 10µl Sperm Blue fixative solution. Samples were stored in fix for between 3min-1hour, after which they were loaded onto a standard microscopy slide with Dabco (Sigma, UK) mounting medium. All slides were assessed within 24 hours of preparation. Samples were observed with an Olympus BX61 microscope under epifluorescence illumination using UV BP 425 and 450-490/LP 515 excitation/emission filters. Spermatozoa were categorised as follows: A; incapacitated (bright fluorescence is identified over the sperm head), B; capacitated (fluorescence over acrosome region with dark post acrosome), C; acrosome reacted (spermatozoa show a mottled fluorescence over the head, fluorescence only in post acrosomal region or no fluorescence on the head). Two slides per sample were evaluated, with at least 100 spermatozoa per slide. This experiment procedure was adopted from (Mattioli et al., 1996), but the CTC concentration was optimised in the laboratory based on our used boar breeds in this experiment. A t-test was used in

order to determine the optimum time for sperm capacitation and ANOVA was used to compare three different capacitation media together. A value of P<0.05 was considered statistically significant.

Compound	Final concentration
Sodium chloride	116.36mM
BSA (FAF) mg/ml	2mg/ml
D-glucose	10mM
Trisma base	19.98mM
Calcium chloride dehydrate	7.55mM
Sodium pyruvate	4.91mM
Kanamycin monosulphate	2.1mM
Potassium chloride	2.98mM
Water	
Carbohydrate	20mM

Table 2.3: Components of capacitation medium.

2.2.7 Sperm chromatin packaging

In order to evaluate chromatin packaging in boar sperm and determine whether there is any correlation between boar sperm morphology and chromatin packaging, chromomycin A3 (CMA3) staining was performed to test for protamine deficiency in sperm (a measure of chromatin packaging). The protocol for this experiment was adopted from (Iranpour et al., 2014).

2.2.7.1 Preparation of fixed sperm samples

Boar semen samples were prepared for performing all relevant experiments by firstly washing in sperm wash (10mM NaCl, 10mM TRIS pH 7.0, 0.58g NaCl/1.21g Tris per 1L), followed by centrifugation at 1,900 rpm for 5 minutes. The supernatant was removed without disturbing the pellet, and the pellet was resuspended in sperm buffer, and repeated three times. Following the final centrifugation, sperm samples were fixed by adding 6ml of ice cold 3:1 methanol: acetic acid drop wise and fixed samples were stored at -20°C. For FISH slide preparation, samples were centrifuged at 1,900 rpm for 5 minutes and 10µl of sample of an appropriate concentration was loaded on a standard microscopy slide following the addition of 10µl of fixative solution. 5ml of fixative solution was added to the semen sample for storage at -20°C.

2.2.7.2 Slide preparation

 Superfrost microscope slides (Thermos Fisher Scientific, USA) were cleaned by immersion in methanol and the underside of each slide was marked with a diamond marker to indicate the location of the sample. The slide was labelled properly, before loading samples onto the slide. The slide was steamed and then 10µl off the sample was dropped onto the marked area, and at the same time 10µl of fresh fixative was added. Then the slide was left to air dry for 1 hour.

2.2.7.3 Chromomycin A3 staining

The positive control used in this experiment were sperm cells decondensed by the immersion of slides in 10mM freshly made DTT (Melford) and 0.4 gr Papain (Sigma, UK) made up in 0.2M tris pH8.6. Slides were immersed for 3 minutes followed by washing in PBS for 5 minutes, before rinsing in dH$_2$O briefly and air drying.

Chromomycin A3 from Streptomyces griseus was dissolved in McIlvane's solution (0.2M disodium orthophosphate heptahydrate, 0.1M citric acid, 10mM magnesium chloride pH 7) at a final concentration of 0.25mg/ml of CMA3. 100µl of this CMA_3 solution was added to each slide and before incubation in the dark for 25 minutes, washing in McIlvane's solution for 30 seconds and air drying in the dark. One drop of Vectashield (without DAPI) was added to each slide and a 24X50 mm cover slip was placed on each slide. Slides were observed using a BX61 Olympus microscope equipped with CCD camera and appropriate filter (460-470nm) under 100 x magnification. Smart Capture software (Digital Scientific) was used to capture images. For each slide 100 nuclei were captured and the percentage of positively stained nuclei was calculated (staining was indicated by bright yellow staining).

Decondensation process on boar sperm nuclei

In order to optimise the decondensation process on boar sperm for performing fluorescence in situ hybridisation experiments and to compare the decondensation process between fertile and sub-fertile groups, several decondensing agents were used. These included; DL-Dithiothreitol (DTT) (Melford), sodium hydroxide (NaOH) and DTT mixed with (0.320g) Papain (Sigma, UK). Two different concentrations of DTT (10mM, 25mM) were dissolved in 0.1M Tris-HCL (pH8) and one slide for high quality boar semen group and one slide of poor quality semen group was immersed for three time periods; 20, 40 and 60 minutes for each concentration of DTT. After this step, slides were washed in 2xSSC for three minutes twice. Subsequently, slides were washed in a cold ethanol series (70%, 85% and 100%) for 2 minutes each in order to dehydrate. Slides were allowed to dry, mounted with Vectashield mounting medium with DAPI (Vector, UK) and covered with a cover slip. Boar sperm decompensation procedure was adopted from (Orsztynowicz et al., 2011)

2.2.7.4 DTT with papain

Slides were immersed in 40ml of 0.2M Tris-HCL (pH8) with 10mM DTT containing 0.320g

Papain (Sigma, UK) for three minutes. Slides were then washed with 2XSSC and cold

ethanol series as explained in the previous section and left to dry counterstaining with

Vectashield mounting medium with DAPI (Vector, UK) (Orsztynowicz et al., 2011)

2.2.7.5 Fluorescence in situ hybridisation (FISH)

Dual colour fluorescence in situ hybridisation (FISH) was performed to estimate aneuploidy

rate on boar sperm. FISH probes for all chromosomes were prepared at the University of

Kent, and also all FISH procedure was performed in this experiment was developed at the

Kent University. This process is described in the following sections.

2.2.7.5.1 LB Agar preparation

20g of LB Broth (Invitrogen) and 20 g agar (Gibco) were added to 1 litre of ddH_2O,

autoclaved at 120ºC and left to cool to 50ºC. 600µl of the antibiotic chloramphenicol

(25mg/ml) (Fluka Biochemika) was added to give a final concentration of 15µg/ml and

approximately 10ml of cooled agar was poured into sterile plastic Petri dishes and allowed

to set overnight before storage at 4ºC.

2.2.7.5.2 LB Broth preparation

20g of LB broth was added to 1 litre of ddH_2O, autoclaved at 120ºC and left to cool to 50ºC.

600 µl of the antibiotic chloramphenicol (25mg/ml) was added to give a final concentration

of 15µg/ml. After that, the Broth was used to inoculate the starter culture for the mini-prep

(Qiagen) (see section 2.4.1.4)

2.2.7.5.3 Preparation of BACs

A disposable sterile pipette tip was inserted into each agar stab containing the BAC clone

and transferred to separate 50ml falcon tubes containing 20 ml of prepared LB Broth

(containing chloramphenicol) and then left to culture overnight in the shaker at 37ºC at

140rpm. A sterile disposable pipette was used to streak 10µl of each sample from falcon tubes into agar plates and left to culture overnight at 37ºC. On the following day, two colonies were taken from the agar plate with a sterile disposable tip and were transferred to 20ml universal tubes including 5ml of LB Broth in glycerol solution (7% glycerol). After that, the tube was left to culture overnight at 37ºC in a shaker at 140rpm. 1ml of each culture was taken and stored at -20ºC.

2.2.7.5.4 Plating out of BACs

A sterile disposable pipette was inserted into the glycerol stock of the BAC clone and used to streak an LB agar plate. Following incubation at 37ºC overnight, agar plates were washed with PBS and colonies were scrapped with a plaster pipette. The solution obtained was centrifuged at 8000 rpm for 3 minutes in order to isolate clones for BAC isolation.

2.2.7.5.5 BAC DNA isolation (Qiagen plasmid mini kit)

After centrifuging as described in the previous section, the remaining pellet was resuspended in 250µl of buffer PI (containing RNase A and lyse blue) to lyse the cells, followed by addition of 250µl of buffer P2 and inverting 4-6 times. Then 350µl of buffer N3 was added to neutralise lysis and samples were instantly inverted again 4-6 times. After this step, samples were centrifuged for 10 minutes at 13000 rpm. The supernatant was removed to a QIAprep spin column and was centrifuged for 60 seconds. Then, 500µl of buffer PB was added to the sample and centrifuged for 60 seconds, and then 750µl of buffer PE was added and centrifuged again for 60 seconds. The flow through was discarded into Virkon and the samples were centrifuged again for a further 60 seconds to remove residual wash buffer. After that the column was transferred to a clean 1.5 micro centrifuge tube. Next, 50µl of EB buffer was added to the column to elute the DNA and left to stand for minute before a further 60 seconds in the centrifuge. Samples were kept at -20ºC in preparation for labelling.

2.2.7.5.6 Probe DNA amplification

DNA concentration was first measured by a spectrophotometer (Nano Drop 8000, Thermo Scientific). 3 µl of each DNA sample were mixed with 27 µl of sample buffer (Genomifi V2, UK) and pulse centrifuged up to 6 rpm. Then the solution was incubated at 95°C for 3 minutes on a PCR block and immediately placed on ice. In a fresh 1.5ml tube, enzyme/reaction buffer was made up and kept on ice; enzyme volume calculated at a ratio of 3µl x the number of tubes x 1.2, and the reaction buffer volume was calculated at 9 x the volume of enzyme. 30µl of this solution was mixed with cooled probe DNA, and after that pulse centrifuging was performed. Then, sample was incubated at 30°C for 1.5 hours in a dry incubator. After incubation, the sample was put in a water bath at 65°C for 10 minutes to inactivate the enzyme and then placed on ice. 60µl of Microbiological Growth water (MBG) was transferred to a fresh 1.5ml tube, then 12µl of sodium acetate/EDTA buffer (50ml of 3M sodium acetate (pH8) and 50ml of 0.5M EDTA (pH8) was added. 300µl of 100% ethanol was added to the sample and mixed gently by inversion. Next the samples were centrifuged for 15 minutes at 11,000rpm, before discarding the supernatant and adding 500µl of 70% ethanol. After that the sample was centrifuged at 11,000 rpm for 2 minutes. Supernatant was discarded again, and the pellet was pulsed in the centrifuge and any remaining ethanol was removed. The tube lid was left open for 2-3 minutes in order to help the evaporation of residual ethanol. 60µl of 10mM Tris-HCl buffer (10mM pH8) was added to resuspend the DNA overnight at 4°C.

2.2.7.5.7 Nick translation

After leaving the sample overnight, DNA concentration was measured and probe DNA was diluted with 10mM Tris-HCl buffer to a volume of 166.5µg/µl. After this stage, DNAse1 (Sigma, UK) from powder stock was dissolved in 1ml MBG H$_2$O and aliquoted into 100µl stocks to freeze at -20°C. Serial dilution (by t aking 1µl DNAse1 stock solution and diluting

in 999µl MBG H$_2$0) was done. 12µl of sample DNA probe was transferred to a fresh 1.5ml tube on ice, and after that 10µl 10xNT buffer, 10µl 10mMDTT, 8µl NucMixA, 1.5µl Texas Red/FTCI, 4µl DNA polymerase I, 1µl DNAse1, 12µl DNA sample and 50.5µl MBG H$_2$Owere mixed and pulsed, then incubated for 1 hour and 40 minutes at 15°C in a water bat h. After that, they were heat inactivated for 10 minutes at 65°C in the water bath, and t hen it was pulsed centrifuged in preparation for loading onto an agarose gel.

2.2.7.5.8 Agarose gel preparation:

1.4% of agarose gel was made with 0.42 g agarose, 30ml 1xTAE/TBE and 1µl SYBR safe (Life technology), and was left to set with a comb in. The comb was removed and 1XTAE/TBE was poured to cover the gel. 4µl loading buffer was mixed with 4µl DNA probe sample, and then 8µl of each mixed sample was loaded into wells alongside a 3µl 100bp DNA ladder (with 4µl loading buffer). The gel was running for 23 minutes at 90 volts. Smears were checked under a UV lamp to confirm fragment sizes < 500bp. After that probe purification was performed as follows.

2.2.7.5.9 Probe purification

Probe purification was done using a QIAquick nucleotide removal kit (QIAGEN). The column could hold up to 1ml. To each probe volume, 10 volumes of buffer PNI (including isopropanol) was added and mixed. After that step, 800µl of solution was transferred to the quick spin column and was centrifuged at 6,000 rpm for 1 minute, and the flow through was discarded. After that, the remaining probe was added to the column and centrifuged for 1 minute at 6,000 rpm. The column was washed with 750µl of PE buffer (containing ethanol) and was centrifuged for 1 minute at 6,000 rpm and the flow through discarded. One further centrifugation at 13,000 rpm for 1 minute was performed. Then the column was moved in to a new 1.5ml centrifuge tube and 100µl MBG water was added and left to stand for 5 minutes

before spinning at 13,000 rpm for 1 minute. Columns were discarded and purified probes were stored at 4°C.

2.2.8 Fluorescence in situ hybridisation (FISH)

In order to estimate aneuploidy rate in boar sperm, dual colour FISH was performed. Sample and slide preparation are described in sections 2.2.3.4 and 2.2.3.2.

2.2.8.1 Pre-hybridisation washes

After preparing slides, they went through decondensing process as optimised previously. Slides were immersed in decondensing solution (0.320 g Papain, 0.155gr DTT (10mM) per 100ml, Tris 0.2 M pH 8.6) for 3 minutes at room temperature. After the decondensing stage, slides were washed briefly in 2xSSC and left to air dry. Slides were washed in 70%, 85% and 100% ethanol for 2 minutes each, air dried and in the meantime, 10µl hybridisation mix was prepared (4µl MBG water, 2µl porcine hyblock, 2µl FITC labelled FISH probe and 2µl Texas red labelled FISH probe), and loaded onto slides on a hot plate at 37°C with 10µl hybridisation buffer (Cytocell, Cambridge, UK). A 24x50mm cover slip was placed on each slide and sealed with rubber cement. Slides were heated on a Hybrite (Thermobrite, UK) for 5 minutes at 75°C (probe denaturation step), then slide were stored overnight in moist chamber at 37°C.

2.2.8.1.1 Post hybridisation washes and detection

On the following day, the rubber cement was carefully removed using tweezers and slides were incubated in 0.4xSSC in order to permit the cover slips to float off. They were then transferred to pre-warmed 0.4xSSC at 72°C for 2 minutes. After this slides were immersed in 2xSSC with 0.05% tween 20 for 30 seconds, then 10µl Vectashield with DAPI (Vectors, UK) was added on each slide and a cover slip (24x50mm) was placed on slides. Slides were kept in the fridge until evaluation. Slides were observed using fluorescence microscopy as

described previously. 4 different breeds (2 animals for each breed) were analysed and 500 spermatozoa were counted per slide.

2.2.8.1.2 Chromosome positioning

This study was performed to investigate whether chromosome positioning on boar spermatozoa is random or non-random, and to see whether there is a difference between fertile and sub-fertile groups. Slides from previous FISH experiments were re-analysed; 150 images of spermatozoa were captured by Olympus BX61 microscope which was equipped with a CCD camera and appropriate filter under 100x objective. Images were captured with Smart Capture 3 software (Digital Scientific, UK). For detecting the position of signals in boar sperm, a custom designed macro (Designed by Michael Ellis, Digital scientific) in image J (available from http://imagej.nih.gov/ij/) based on that of Croft et al. (Croft et al., 1999) which is capable of counting signals was utilised. Chi-squared statistical tests were performed automatically.

2.2.9 Telomere detection in boar sperm (FISH)

In order to assess telomere distribution in sperm nuclei, fixed semen samples were used as described in section (2.1.1) and slides were prepared for FISH according to section 2.2.3.2. In this experiment four fertile breeds and four sub-fertile breeds were used and per breed two animals were studied. After preparing boar sperm slides according to previous sections, DTT treatment was performed (Sigma, UK) using 10mM DTT, 0.320 g papain dissolved in 0.1M tris-HCl pH8 for 30 minutes in the dark. This experiment procedure was adopted from (Hazzouri et al., 2000) but the experiment was optimised according to the used boar breeds.

2.2.9.1 Pre-hybridisation washes

Following decondensation, slides were immersed in PBS solution for 15 minutes and fixed

in 4% formaldehyde (in PBS) for 4 minutes. Slides were washed in PBS twice for 2 minutes,

dehydrated in cold ethanol series for 2 minutes each in (70%, 85% and 100%) and were left

to air dry. Subsequently, 15µl of PNA probe (Panagene, UK) in hybridisation buffer was

added to each slide and slides were covered by a cover slip. Slides were denatured for 5

minutes at 80ºC and incubated in the dark at room temperature for between 30 minutes and

2 hours.

2.2.9.1.1 Post hybridisation washes

After incubation, Slides were immersed in washing solution I (PBS/0.1% tween20) in order

to remove coverslips. Following this, slides were washed in washing solution I for 20 minutes

at 57ºC, then in washing solution II (2XSSC/0.1%tween-20) for 1 minute at room

temperature. A few drops of Vectashield mounting medium with DAPI were placed on slides

and a coverslip (24x50mm) was placed on each slide. Slides were kept in the fridge until

evaluation.

2.2.9.1.2 Telomeric signals analysis

Telomere signal positions were identified in at least 200 sperm cells from four breed of fertile

boar sperm and four breeds of sub-fertile boar sperm using a custom designed macro

(designed by Michael Ellis, Digital Scientific) in Image J. This macro programme is capable

of splitting the area of the nucleus into five concentric rings of equal area and detecting the

proportion of telomere signal within each area, relative to the total telomere signal. A chi-

squared test was used to test the hypothesis that signals are distributed non-randomly within

the sperm nucleus.

2.2.10 Impact of biopsy on embryo morphology

This retrospective study was performed by analysing time-lapse images of embryos from patients at the Assisted Conception Unit at Guy's and St Thomas' Hospital, London.

2.2.10.1   Patient information

Patients with known outcome such as fetal heart beat (FHB), live birth (LB), failed implantation and embryo transfer resulting in a singleton pregnancy, and following ICSI were included in this study. Those embryos without any information on their outcome were excluded. The female age range in this study was from 25 to 45 years (mean± SD 35.7± 4.2).

2.2.10.2   Ovarian stimulation and oocyte retrieval

Patients underwent a variety of ovarian stimulation methods such as long down regulation protocol (Supracur Hoechest, Germany), a short antagonist protocol (Cetrotide Serono) and recombinant follicular stimulating hormone (R-FSH) (Gonal-F; Merck Serono). FSH doses ranged from 150 to 600 IU per day according to the patient's ovarian response. Oocyte retrieval was performed using ultrasound-guided puncture of ovarian follicles 36 hours later.Ovarian stimulation procedure was performed by specialised IVF nurse at St Thomas guys hospital reproductive centre (London,UK)

2.2.10.3   Embryo preparation

After performing oocyte retrieval, oocytes were fertilised by ICSI. ICSI fertilised embryos were placed in the Embryoscope (Unisence Fertilitech, Denmark) immediately after injection and were cultured for 5 or 6 days. They were placed on an individual sterile embryoslide (Unisense Fertilitech, Denmark) which has a capacity of 12 embryos with 25µl media per well. IVF™ Plus (Vitrolife, Sweden) was used as the fertilisation media and was placed in each well of the Embryoslide on the day of insemination. Embryos were cultured in G-1™ (Vitrolife, Sweden) media from day 1 to day 3 of embryo development and day 3 onwards in

G-2^{TM} Plus (Vitrolife, Sweden). To permit temperature stabilisation, Embryoslides were prepared one day before use. After loading embryos, the Embryoslides were placed directly into the Embryoscope and image acquisition started immediately. In order to change the embryo culture medium on days three and five, the pause icon was clicked, 20µl of medium was removed and 20 µl of pre-equilibrated fresh culture medium was added. For each well the Embryoscope was able to determine which focal planes yielded the most informative images; this process took only four minutes. After this, the Embryoscope continued with image acquisition from each well in multiple focal planes. All this process was performed by embryologists at St Thomas guy's hospital reproductive centre (London, UK).

2.2.10.4 Embryoscope software

All data produced are viewed and analysed using Embryo viewer software (Unisense Fertilitech, Denmark). It was possible to observe images of all embryos in the embryo slide culture dish at once or individually. Images could be enlarged for observation where annotation tools were available. These tools included: Recording the appearance and disappearance of pronuclear (PN), cleavage times, fragmentation, blastomere evenness or unevenness, multinucleation as well as morula and blastocyst formation times (see figure 2.2). Events were annotated by clicking the plus or minus sign in the upper left corner of the annotation box until the relevant number of cells was displayed. A black vertical line was revealed in the division chart to demonstrate the time at which the cell division took place. The annotation was performed by clicking on the field which demonstrates the number of cells (upper left corner on annotation screen). Annotation was done as follows: 1, 2, 3, 4, 5, 6, 7, 8, 9+ for the number of cells; start of compaction; morula, start of blastulation (B), expanded blastocyst (EB), hatching blastocyst (HB) and dead for atretic embryos. When annotation was performed, a value was inserted in the list of annotation variables. The time

(hours since fertilisation) was automatically included by the software. Table 2.4 demonstrates the annotation variables which were used in this research. This annotation procedure was established by Rebecca Gould (University of Kent, UK) at the Bridge Fertility Centre (London, UK). When annotation was performed, data was exported to an Excel spreadsheet and a Mann-Whitney U test was performed for statistical analysis.
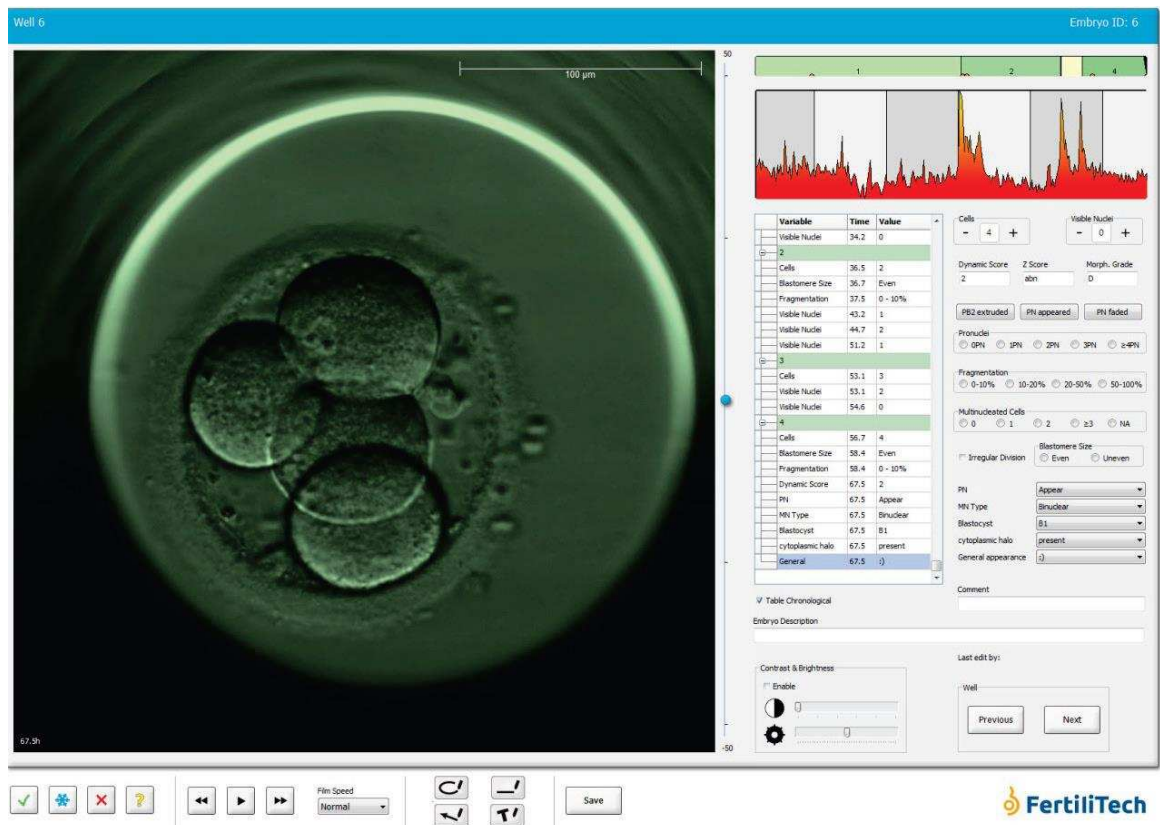
Figure 2.2. Annotation parameters that were considered during the embryo annotation process. Blastomere activity is displayed on the right. Annotation parameters such as appearance and disappearance of pronuclei time, number of cells, cleavage times, fragmentation, blastomere size, multinucleation, and irregular division, time of morula formation and time of blastocyst formation are displayed here.

Annotation variables	Comments
PN appearance Number of PN visible 0PN 1PN 2PN 3PN >4PN	Time of its appearance was recorded
Number of PN visible	17 hours- or the closest point
PN fading	Time of PN disappearance was recorded
One time point before PN fading was checked	Number of visible PN was recorded
Further checking was performed at 24 hours-or the closest point	To make sure if early cleavage sign was observable and also PN fading performed
First division. Formation of two distinct new blastomeres from a single blastomere (each blastomere has a clear plasma membrane)	Cell number, blastomere size (evenness or unevenness), fragmentation percentage (0-10%, 10-20%, 20-50%, 50-100%), and time of occurrence of first division were recorded. Irregular division box was scored if it was observed.
Further check was done at 27 hours-or the closest point	Cell number at this time point was recorded
Second division (formation of three cells), when two cells divided in three cells	Time of second division was recorded
Halfway of first and second division was considered	To determine if multinucleation was observed (number of multinucleated blastomers was recorded). Blastomere size (evenness/unevenness), fragmentation percentage according to its category was recorded at this time point.
Formation of third (t3) division	Blastomere size, fragmentation percentage according to its category was recorded.
4th division t4 (4 cells divided to 5 cells)	Time of appearance of 4th division was recorded
Halfway of third and fourth division was considered	To determine multinucleation as described before
5th division t5 (5 cells divided to 6 cells)	Time of appearance of 5th division was recorded
6th division t6 (6cells divided to 7cells)	Time of appearance of 6th division was recorded
7th division t7 (7cells divided to 8)	Blastomere size, fragmentation percentage according to its category was recorded.
8th division t8 (8cells divided to 9)	Time of appearance of 8th division was recorded
Halfway of 7th division and 8th division was considered	Annotation was done based on described category
Start of compaction (when the plasma membrane of the blastomeres becomes unclear before the cells start to compact)	Time of start of compaction was recorded
Morula stage	Time of Morula stage formation was recorded
Start of blastulation	Time of formation of cavity was recorded
Early blastocyst or BC1 (blastocoel less than half of the blastocyst)	Time of appearance of this event was recorded
Blastocyst or BC2 (blastocoel more than half of the blastocyst)	Time of appearance of this event was recorded
Full blastocyst BC3 or B in annotation box (blastocoel fills the blastocyst)	Time of appearance of this event was recorded

Expanded blastocyst (EB) the embryo is large and Zona is thin	Time of appearance of this event was recorded
Hatching blastocyst (HB) Zona pellucida starting to come out of the shell	Time of appearance of this event was recorded
Hatched blastocyst (Zona pellucida out of the shell)	Time of appearance of this event was recorded

Table 2.4. This table illustrates the many annotation variables which were considered to annotate embryos for this research from pronuclear stage to hatched blastocyst stage.

2.3   Statistical analysis

Statistical analysis was performed in this research are follow as:

2.3.1   Chi-squared statistic test:

To statistically analyse the impact of freezing on boar sperm morphology, and to compare semen morphological features between high quality and poor quality boar semen groups chi-squared test was used. (Section 3.3.2, 3.3.3). This test was performed to statistically analyse the impact of sperm concentration on fertilisation rate, and also for studying whether there is correlation between boar sperm capacitation rate and fertilisation potential, analysis using Chi squared was used to statistically analyse the capacitation rate differences in both groups of high and poor quality boar semen (section 4.2.1, 4.2.4, 4.2.5). In section 5.3.1, in order to test hypothesis that the sperm chromatin packaging is significantly different in the poor quality semen group compared the high quality boar semen group as assayed by CMA3 staining, Chi squared test was performed (Specific aim 3a). Chi squared test was used to statistically analysed whether telomere distribution and chromosome positioning is random or non-random in both groups of high and poor quality boar semen. This statistic was performed to statistically analyse the distribution of abnormal number of chromosomes in high quality and poor quality boar semen groups (section 5.3.2, 5.3.3). In section 6.2.2, chi squared test was used to compare blastocyst and hatching blastocyst rate and treatment outcome in patients

that opted for embryo biopsy at the cleavage stage with that of patients that opted for

embryo biopsy at the blastocyst stage (specific aim 4b). Results were considered

significant for p ≤ 0.05.

2.3.2 Independent t-test:

Independent-test was performed in this thesis when our data were distributed normally.

In section 4.2.3 t-test was used to statistically compare the capacitation rate in three

defined capacitation media.

In section 6.2.1, to investigate Timing of various developmental stages among embryos

biopsied at cleavage or blastocyst stage, Mann Whitney U test were used to determine

differences in two groups. As our data, did not have normal distribution, Mann Whitney U

test was selected. Results were considered significant for p ≤ 0.05.

3   Specific aim 1: Estimating Boar semen quality for in vitro fertilisation commercial application

3.1   Background

Boar semen quality is considered to be an important factor in pig IVF (Gil et al., 2008) interestingly, evidence shows that boar fertility largely depends on the breed line. Additionally, several epigenetic factors are involved in the incidence of sperm defects, including acrosome defect, head defects, midpiece abnormalities and tail defects (Broekhuijse 2011; Wimmers et al., 2005). Furthermore, semen quality of a single boar may significantly change over a short period of time due to seasonal patterns (Sancho et al., 2004), environmental impacts (Murase et al., 2007) and infection status (Bussalleu et al., 2011). Several traditional methods have been suggested to evaluate semen quality in order to increase pig IVF success rates, including motility and morphology evaluations (Sancho et al., 2004). Of these, motility assessment has been considered as one of the most common methods owing to its simplicity and inexpensive nature (Sancho et al., 2004). Indeed, results from Gadea and colleagues suggest that motility evaluation is strongly correlated with spermatozoon membrane intactness and piglet farrowing rates (Gadea et al., 2005). Having said this however, others have suggested that motility assessment alone cannot be an effective factor in determining semen quality, and that when combined with other tests, the predictive potential of sperm motility on semen quality is improved (Yeste et al., 2010). Similarly, sperm morphology assessment has been considered as another important, inexpensive method to evaluate semen quality. In agreement with this, Broekhuijse and colleagues (2011) showed that a high rate of morphologically abnormal spermatozoa has a negative effect on fertility (Broekhuijse et al., 2011). However, there is no clear agreement in the literature over the threshold at which the percentage of abnormal spermatozoa should be adopted to distinguish between good and poor semen samples. Several studies suggest

a minimum of 70% normal morphology can be considered as a good semen sample (Shipley et al., 1999), while others recommend 80% or 85% (Feitsma, 2009 Orsztynowicz et al., 2011).

One factor that is known to impact sperm morphology is freezing, a necessary process for the preservation and global distribution of semen samples. Evidence shows that following freezing, the spermatozoa plasma membrane is damaged (Guthrie et al.,2005) and the acrosome and tails are adversely affected (Ozkavukcu et al., 2008). However, such studies are extremely limited, particularly in the context of freezing. This chapter therefore aims to explore these issues.

3.2   Specific aims of this chapter

The detailed specific aims of this chapter were as follows:

Specific aim 1a: To produce a working classification system for boar sperm morphology

Specific aim 1b: To compare semen morphological features between fertile and sub fertile boars

Specific aim 1c: To test the hypothesis that sperm cryopreservation impacts on sperm morphological features.

3.3 Results

3.3.1 Specific aim 1a: To produce a working classification system for boar sperm morphology

Using Sperm Blue stain (Microptic), a detailed morphology evaluation was performed on a total of 49358 spermatozoa from 20 boars of different breeds. The WHO 2010 guidelines were used as a basis to categorise semen morphological features. Abnormalities were found in either the head, midpiece or tail regions, or various combinations of the three. This is illustrated in Figure 3.1. Table 3.1 describes the features for each cell represented in Figure 3.1, which formed a basic guide to assist with the recognition of anomalous morphological features.
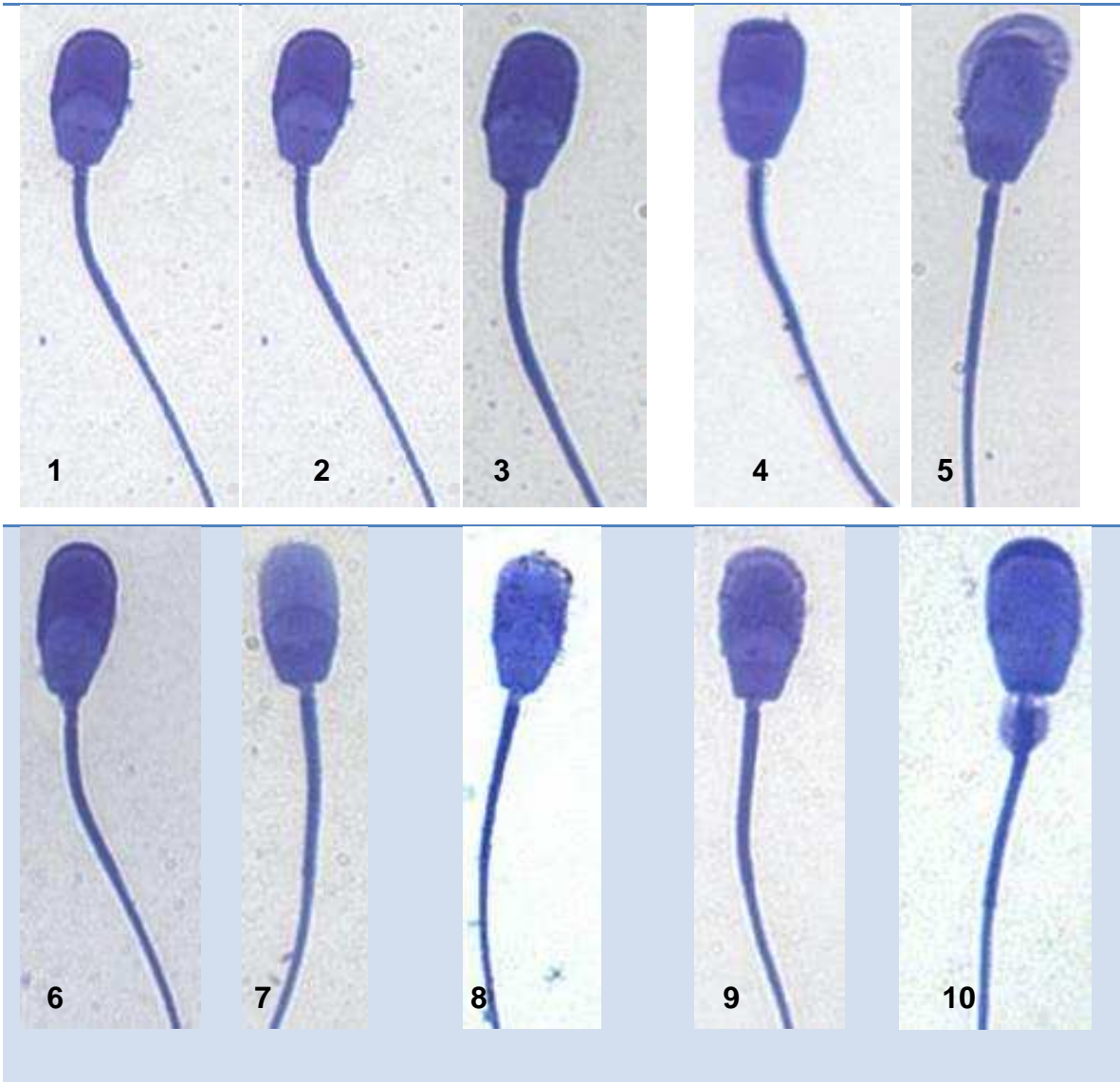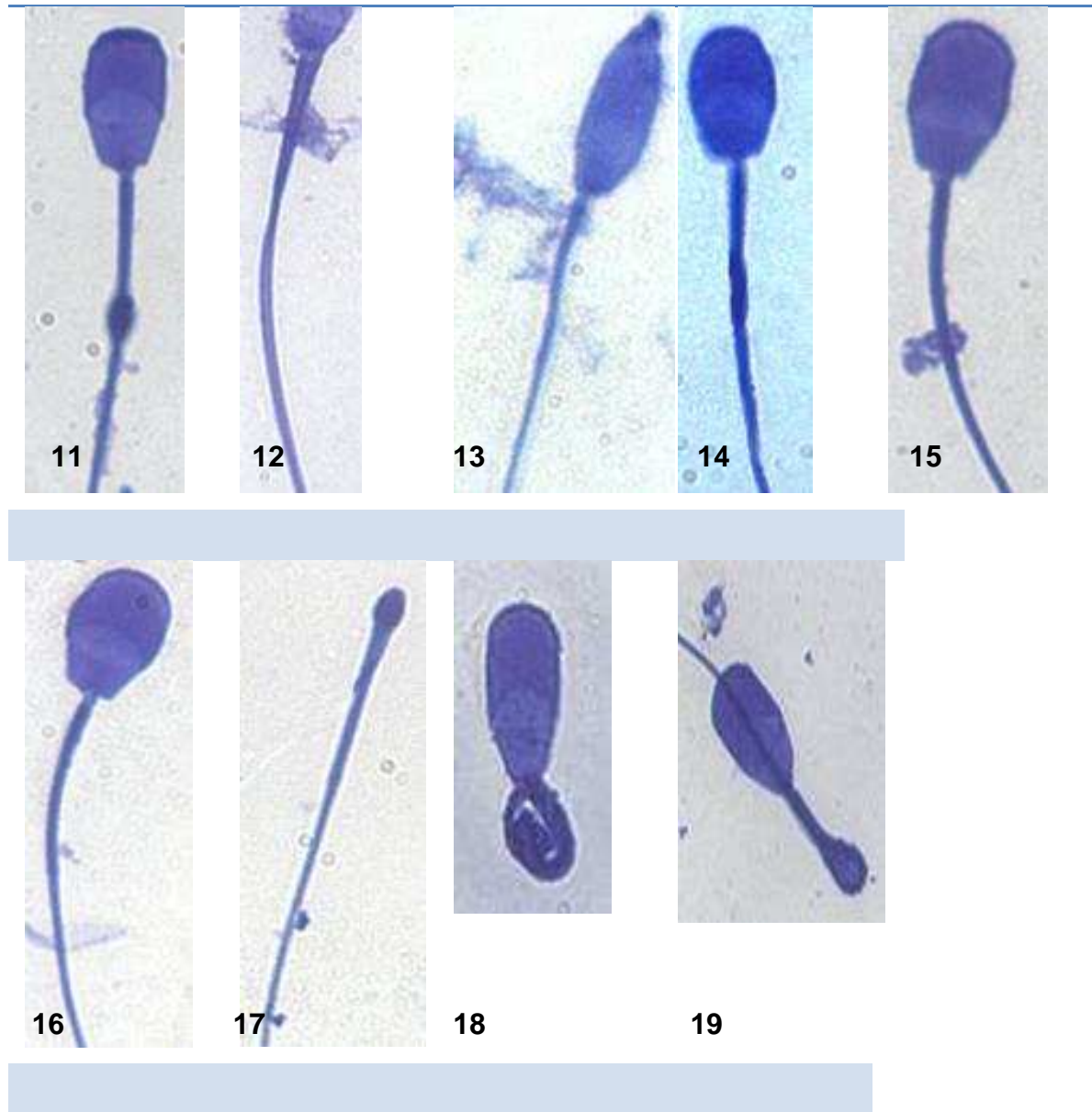
Figure 3.1. Morphological features of boar spermatozoa. Sperm are stained using a Sperm blue kit and photographed at 1,000x magnification. The first three cells are normal (1-3), while the other images indicate boar spermatozoa with abnormal morphological features (4-21). Please see supplementary Table 3.1 for a more detailed description of these morphological anomalies.

Sperm	Abnormality	Comment
1	-	Normal, typical form
2	-	Normal, typical form
3	-	Normal, typical form
4	Acrosomal	Thick and irregular acrosomal edge
5	Acrosomal	Detached acrosome
6	Acrosomal	Acrosome lifted, not tightly adherent
7	Acrosomal	Acrosome cap missing
8	Acrosomal	Vacuolated acrosome
9	Acrosomal	Vacuolated acrosome
10	Retained droplet	Retained proximal droplet, immature cell
11	Retained droplet	Retained distal droplet, immature cell
12	Multiple tails	Two tails
13	Head	Elongated head
14	Head	Pear shape head
15	Head +droplet	Large, pear shaped head; distal cytoplasmic droplet
16	Head	Pear shaped head
17	Head	Pinhead
18	Tail	Coiled tail
19	Tail	Folded tail

Table 3.1. Abnormal morphological features found in spermatozoa as represented in Figure 3.1. For each spermatozoon, the type of abnormality is described. The first 3 spermatozoa indicated no abnormalities and therefore are considered as controls.

3.3.2 Specific aim 1b: To compare sperm morphological features between fertile and sub fertile boars

Boar spermatozoa morphology assessment was performed for both high and poor quality boar sperm groups and in each group, there were 4 breeds such as Hampshire, Landrace, Pietran and Large White. Four animals for each group were used for the work described under specific aim 1a. On average, the known high quality boar semen group displayed approximately 60% normal morphology while the known poor quality boar semen group showed 39% normal morphology. The first and second most common morphological abnormalities in the fertile group were acrosome abnormalities (17.4%) and head shape abnormalities (8.2%) respectively (Figure 3.2). However, in the poor-quality group, the first and second most common sperm morphological abnormalities were acrosome abnormalities (19%) and coiled/folded tail (17.4%) respectively (Figure 3.3).
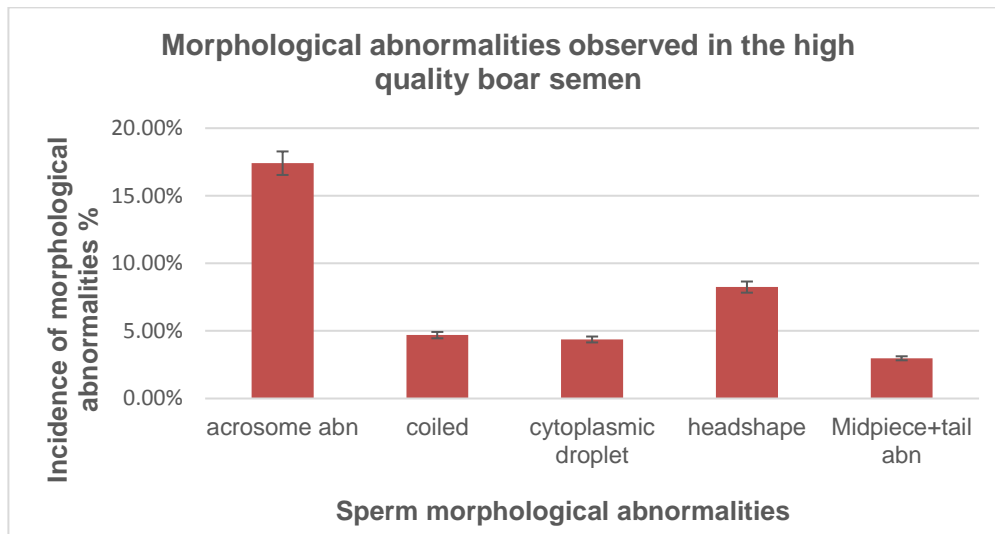
Figure 3.2. Sperm morphological abnormalities observed in the high-quality semen group (in total 11 animals, four breeds). The most common sperm morphological abnormalities among the fertile group were acrosome abnormalities with a frequency of 16.9%, followed by head shape abnormality with a frequency of 9.9%.
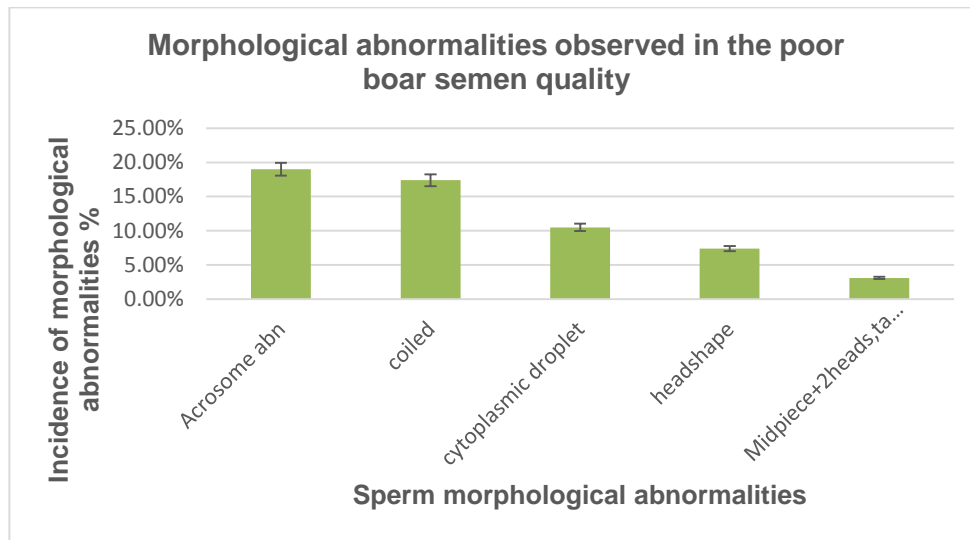
Figure 3.3 Morphological features among sperm in the poor-quality group (9 animals, 4 breeds). The most common sperm morphological abnormalities among the sub fertile group were acrosome abnormalities with a frequency of 19% followed by coiled/folded tail with a frequency of 17.4%.

Chi-squared analysis revealed that poor-quality boar semen had significantly lower levels of normal morphology compared to the high-quality semen group across all types of morphological parameters assessed, with the exception of acrosome or head vacuolisation. Figure 3.4 and table 3.2 illustrate the comparison of semen morphological feature between high quality and poor quality boar semen groups.

Morphology assessment
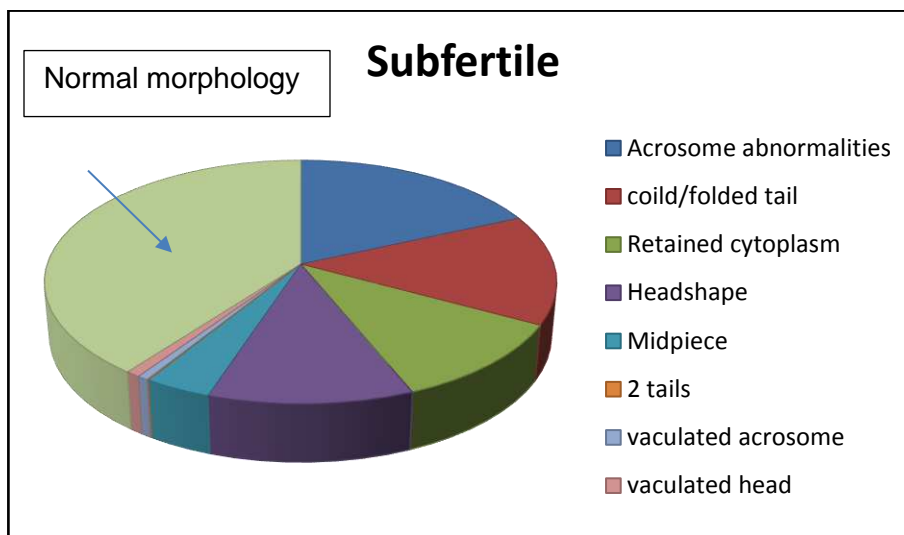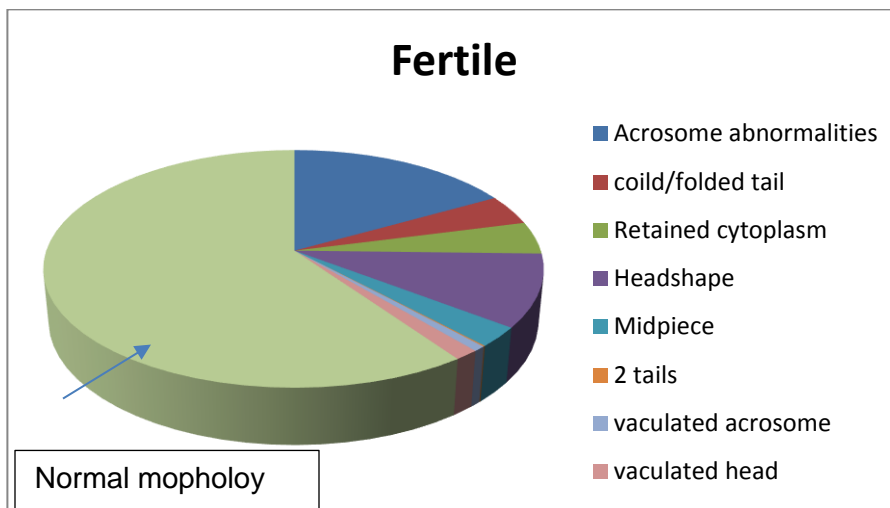
Figure 3.4. Comparison of semen morphology characteristics between the high-quality (top) and poor-quality (bottom) semen groups. Features detailed in table 3.1 were represented in 9 categories. The percentage of normal forms varies considerably between the two groups; however, acrosome abnormalities appear to be evenly distributed.

Sperm morphological Feature	Numbers Observed		Chi square	df	P-value
	High boar semen quality	Poor boar semen quality			
Normal	14482	6292	5414.97	1	0.00E0
Acrosome shape/size	637	946	58.735	1	1.80E-14
Lifted acrosome	1519	1238	33.502	1	7.12E-09
Detached acrosome	1737	1548	13.878	1	0.000195
Absent acrosome cap	593	1394	326.334	1	6.04E-73
Coiled/folded tail	966	5016	3036.67	1	0.00E
Distal droplet	988	1944	318.822	1	2.61E-71
Proximal droplet	611	1269	231.305	1	3.10E-52
Head shape/size	2956	2509	46.305	1	1.01E-11
Midpiece abnormality	735	929	21.214	1	4.11E-06
Multiple head	9	11	0.176	1	0.672
Multiple tails	25	15	2.612	1	0.106
Vacuolisation	475	514	1.164	1	0.281

Table 3.2. Boar sperm morphology evaluation in fertile and sub fertile groups. Results were compared for statistical significance using a Chi-squared test.

3.3.3 Specific aim 1c: To test the hypothesis that sperm cryopreservation impacts on sperm morphological features

In order to study the impact of freezing on boar spermatozoa morphology, 2,000 spermatozoa from 2 fertile Duroc boars were collected and evaluated both before and after freezing. Results showed that 56.7% (fresh) and 26.3% (frozen) were morphologically normal. Acrosome abnormalities were common among both fresh and frozen spermatozoa (29.5% and 42.4% respectively). The second most common abnormality among fresh and frozen spermatozoa was head shape abnormalities (8.7% and 20.1% respectively); see Figures 3.5 and 3.6.
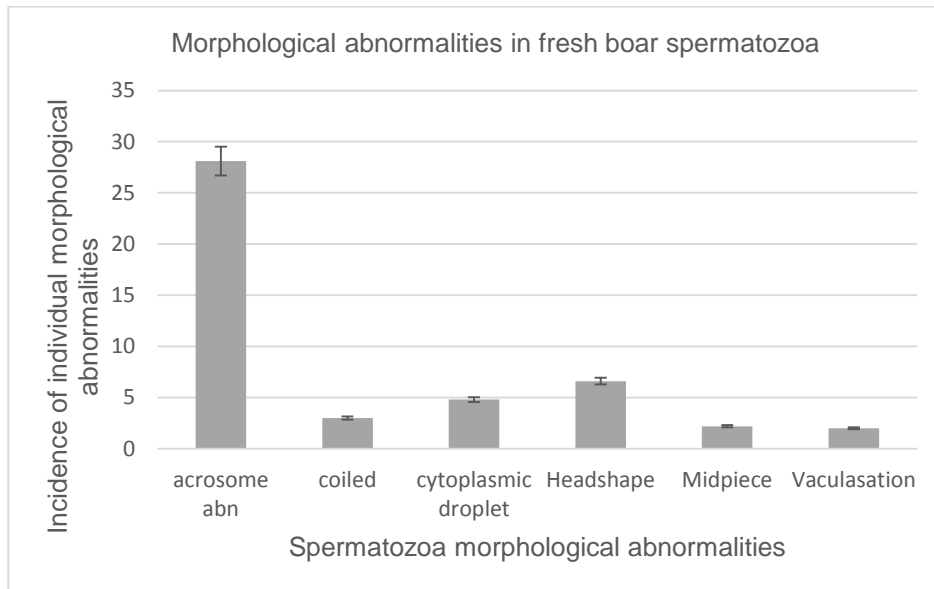
Figure 3.5. Morphological abnormalities in fresh boar spermatozoa (n=2000) before freezing. The most common morphological abnormality among fresh spermatozoa was acrosome abnormalities with a frequency of 28.1%. The second most common abnormality was head shape abnormalities with a frequency of 6.6%
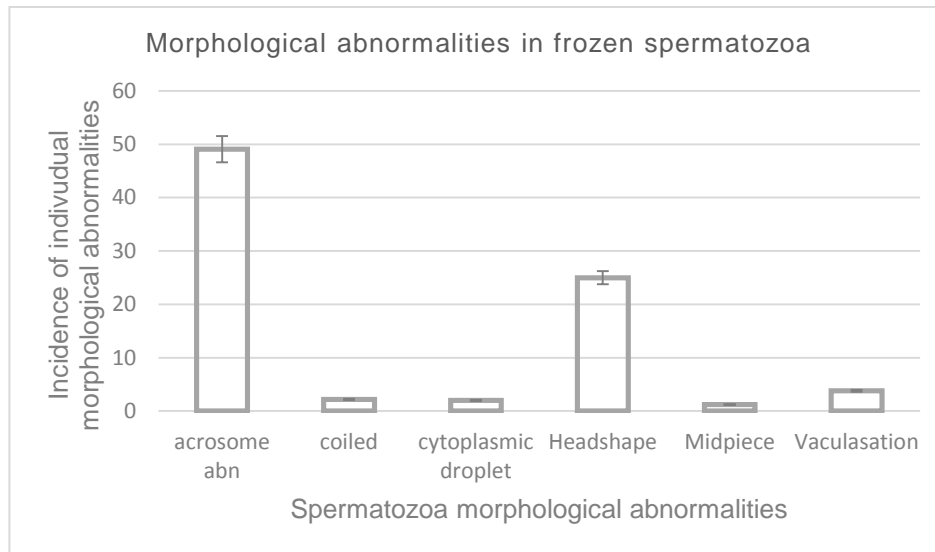
Figure 3.6: Morphological abnormalities among frozen spermatozoa (n=2000). The most common morphological abnormality in frozen spermatozoa was acrosome abnormalities with a frequency of 49.1%. The second most common morphological abnormality was head shape abnormalities with a frequency of 25%.

In order to compare the impact of freezing on sperm morphology, morphological abnormalities incidence between fresh and frozen spermatozoa were compared using a Chi-squared test. Table 3.3 indicates both the raw data and results of the statistical analysis. Results show that fresh spermatozoa had significantly higher percentages of normal morphological features (p <0.05). Overall, frozen spermatozoa showed significantly higher acrosome abnormalities, head shape abnormalities and acrosome vacuolisation than fresh spermatozoa (P <0.05). However, no significant difference was observed in coiled/folded tail, and head vacuolisation between the two groups of fresh and frozen. Interestingly, a higher proportion of sperm with retained cytoplasmic and midpiece abnormalities were present in fresh rather than frozen spermatozoa. This is outlined in Figure 3.7.

	Numbers Observed				
Sperm morphological Feature	**Fresh semen**	**Frozen semen**	**Chi square**	**df**	**P-value**
Normal	1121	801	167.778	1	2.25E-38
Acrosome shape/size	51	64	0.187	1	0.6650
Lifted acrosome	453	276	83.97	1	5.03E-20
Detatched acrosome	164	852	7.481	1	0.0063
Absent acrosome cap	140	111	9.408	1	0.0022
Coiled/folded tail	96	90	2.151	1	0.1431
Distal droplet	68	30	21.304	1	3.92E-06
Proximal droplet	128	42	59.085	1	1.51E-14
Head shape/size	240	612	133.1	1	8.68E-31
Midpiece abnormality	103	36	44.162	1	3.02E-11
Vacuolisation	69	135	13.330	1	0.0003

3Table 3.3. Boar spermatozoa morphology before (n=2000) and after freezing (n=2000). Semen features between fresh and frozen samples were compared by using a Chi-squared statistical test.

Fresh vs. frozen boar spermatozoa morphology evaluation
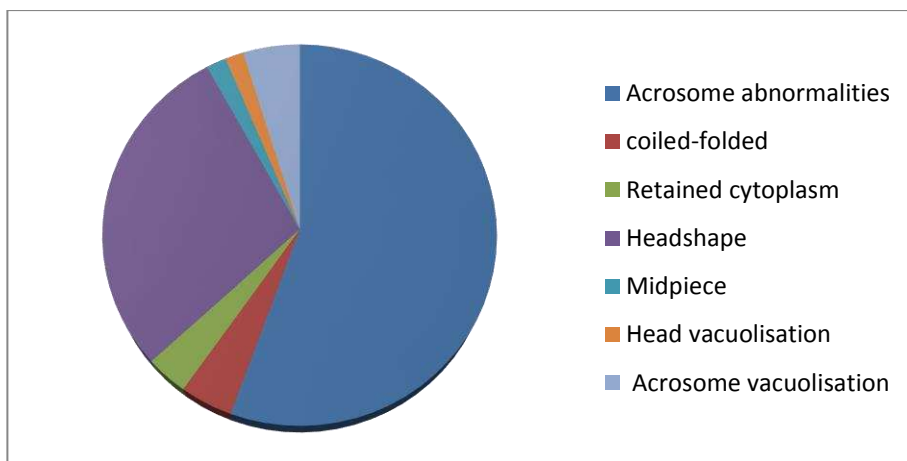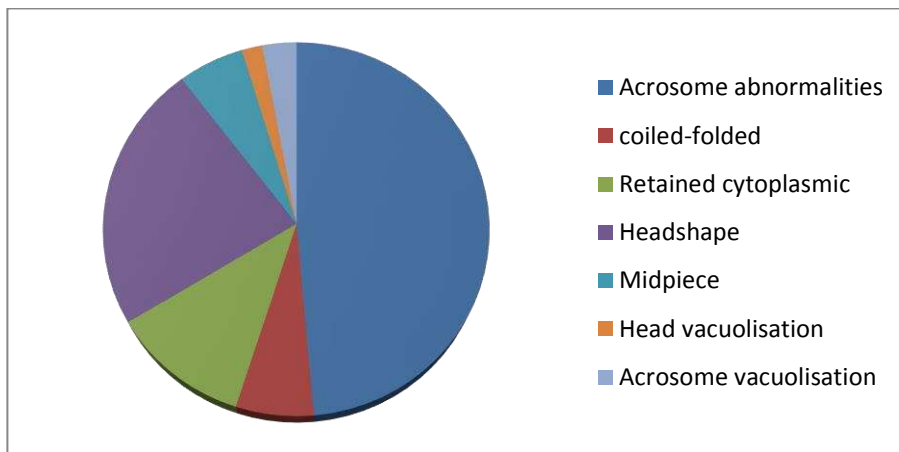
Figure 3.7. Comparison of semen morphology features between fresh (top) and frozen (bottom) groups. Detailed morphological abnormalities in table 3.3 were categorised into 9 categories. The percentage of normal forms appears to be significantly different across the two groups.

3.4 Discussion

3.4.1 To produce a detailed classification of boar semen morphological abnormalities (specific aim 1a).

Current published literature presents some confusion with respect to boar semen abnormality classification and no clear standards exist (Bonet et al., 2012). To the best of my knowledge therefore, the study presented here is the first attempt to develop full criteria for the evaluation of morphological features in boar sperm based on the WHO laboratory manual 2010, for the assessment of human sperm.

As discussed earlier in section 3.1, inter-breed differences in sperm morphology implicate difficulties when trying to standardise the morphological assessment thresholds of high quality boar semen boars (Smital et al., 2009) and an interesting further study would be to determine whether there is a difference in sperm morphology in different breeds. Nonetheless, this work acts as a starting point for a more comprehensive, breed-specific study. Indeed, our data is complemented by appropriate field records of boar farrowing rates, and therefore could supply indications to infer morphology aberration thresholds for each breed. A similar approach was applied before with success (Xu, 1998; Gadea 2004; Gadea et al., 2005).

3.4.2 To test the hypothesis that fertile and sub fertile groups significantly differ in morphological features

Results from this study indicate that some morphological abnormalities (such as acrosome detachment, absence of acrosome and vacuolisation) may be regarded as physiological at the levels encountered in both groups of high and poor quality boar semen. According to previous studies, a threshold of 70% normal forms is considered as a good quality semen sample and a value below this threshold is considered as poor and is therefore discarded (Dominiek 2011;Bonet et al., 2012). In our sperm morphology evaluation experiment,

however, even the boar with the best morphology only achieved 66.5% normal morphological features and the average result for the high-quality semen group was 58.5%. There are several reasons that might explain this observation in the high-quality semen group: firstly, most semen samples evaluated were produced during the spring and summer seasons. Seasonal patterns in boar semen quality have been well documented before, and it has been suggested that semen samples produced during spring and summer have lower quality and quantity due to higher environmental temperatures in comparison to those produced during autumn and winter (Pokrywka 2014; Sancho et al., 2004). Also, a more recent study by Lipensky and colleagues in 2011 indicated that season has the adverse effect on sperm morphology and considerably impact boar semen quality (Frydrychova et al., 2011). Suriyasomboon research group in 2005, reported that high temperature has a negative impact on sperm morphological features, particularly the rate of abnormal head shape may increase in high temperature. The same research group proposed that high humidity may increase the rate proximal cytoplasmic droplet (Suriyasomboon et al., 2005)

In addition, temperature changes during transport from the farm to our laboratory may have had detrimental effects on semen quality. Since spermatozoa are sensitive to alterations in temperature, it is imperative that samples are maintained at 17°C during transit in order to avoid temperature shock. Indeed, a negative impact of high temperature on sperm morphology has been shown in previous research (Lopez Rodriguez et al., 2012). Alternatively, it is possible that our own scoring system is stricter than that implemented by others. Scoring sperm morphological features is highly operator dependent and may be considerably different in different laboratories. Finally, it is of course possible that a proportion of the abnormalities observed can be attributed to errors made during fixing and staining procedures. One possible explanation for the observation of reduced sperm morphology in the poor-quality boar is the presence of bacteria in the semen; previous

evidence has shown that this causes reduced semen quality (Bussalleu et al., 2011). Semen

from healthy boar normally does not have bacteria, however, the skin and hair of the boar

contain bacteria, as do the collection environment which can contaminate the collector's

hand or collection container (Gradil et al., 1991). Therefore, the content of bacteria in semen

extender solution increases after storage of semen for a few days. In our research, some of

our semen sample were stored at 17C° 2-5 days after collection, this issue may impact on

boar sperm morphological features. Although antimicrobial agents are usually added to the

semen extender, in some cases there may be resistance to these treatments which may

negatively impact on semen quality and decrease fertilisation rates (Sone et al., 1990). It

has been suggested that E. coli bacteria attaches to sperm surface through mannose-

binding structures causing damage to the sperm plasma membrane (Wolff et al., 1993). In

line with this, presence of bacteria has previously been shown to disrupt the acrosome,

membrane viability and midpiece of sperm (Kuster et al., 2016).

3.4.3   The impact of freezing on boar spermatozoa morphology

In the pig breeding industry, the use of frozen–thawed boar semen samples for artificial

insemination and in vitro fertilisation is largely limited due to shortened life span of

spermatozoa and lower fertilisation rates leading to variable pregnancy rates (Knox et al.,

2015). Similarly, freezing of semen samples has been reported to lower sperm motility

leading to reduced fertilisation rates (McNamara e et al., 2013). It is thought that this is

largely due to an increased sensitivity to cryopreservation in boar sperm when compared to

human and bovine sperm (Guthrie et al., 2005). In keeping with these findings, results from

our study identified that the process of freezing significantly impacts boar sperm

morphology. Our results showed that overall morphology was highly significantly reduced in

frozen compared to fresh sperm samples from animals of the same breed. More specifically,

the number of acrosome abnormalities, head shape abnormalities and presence of vacuoles

were significantly higher in frozen semen samples compared to fresh samples. It is likely that this can be explained by the high sensitivity of boar sperm to cold shock, which is thought to be cause by the unique composition of phospholipids and cholesterol of their plasma membrane (Watson et al., 1995). During the process of freezing, semen samples are cooled down to below 15 $^{\circ}$C, then to 5°C before a rapid temperature decrease to -100°C. It has been suggested that maintaining boar semen samples at 17°C for between 16 and 24 hours before freezing allows the semen sample to develop resistance to cold shock (Watson, 1995 #489). However, in our study, semen was collected and frozen on the same day after maintenance at 17°C for only a couple of hours. Therefore, it is possible that cold shock may have impacted on acrosome and membrane morphological features as observed in our results. Furthermore, glycerol is added to semen when samples are prepared for freezing. It is well-known that glycerol is highly toxic and can damage the sperm membrane, leading to decreased fertility. It has been suggested that a reduced concentration of glycerol may improve semen quality during the freezing process (Muiño-Blanco et al., 2008) however more recent research indicates that even low concentrations of glycerol can negatively impact on boar sperm motility and morphology. As an alternative, others have recommended use of Trehalose as this is a non-permeable cryoprotectant and a non-reducing disaccharide known to stabilise proteins and biological membranes. As such, Trehalose may improve boar sperm quality during the post-thaw process (Athurupana et al., 2015) In future studies, it would be interesting to investigate the impact of the use of Trehalose over glycerol on sperm morphology in comparison to fresh semen samples.

3.5   Conclusions

In conclusion, we believe to have successfully developed a series of tests that may predict boar semen quality without the requirement for breeding tests. Semen morphological evaluation offers a quick and cheap alternative in order to identify good quality semen

samples. In addition, our results suggest that, to preserve semen quality, extra care is needed during the freezing process. It is possible that replacing glycerol with Trehalose may decrease the toxicity of the semen extender and it would be interesting to investigate such effects in future studies.

4 Specific aim 2: The impact of boar sperm concentration and capacitation on fertilisation rate

4.1 Background

In humans and animals alike, several in vitro methods have been designed for the analysis of sperm fertilisation ability in order to predict male fertility (Xu et al., 1998). In 1990, Bavister and colleagues were one of the first groups to introduce such techniques by assessing sperm morphology and motility, in addition to testing oocyte penetration (Bavister et al., 1990). As mentioned in before, routine techniques in most AI centres and IVF laboratories for the analysis of boar semen quality involve examination of sperm morphology, motility and concentration (Barth 1992; Buckner, 1954; Linford et al., 1976). Previous results have shown that use of a sperm concentration of $1x10^6$ sperm/ml results in high penetration rate, however the incidence of polyspermy is increased (Ding et al., 1992). In light of this, it has been suggested that a decrease in boar sperm concentration may reduce polyspermy and improve penetration rates. One of the aims of this chapter therefore, is to determine the impacts of decreasing boar sperm concentration on fertilisation potential.

In order to acquire the ability to penetrate and fertilise the oocyte, the spermatozoa must undergo acrosome activation in a process known as capacitation. (Yanagimachi, 1994 #541). in vitro fertilisation experiments have achieved capacitation in chemically defined media (Cohen-Dayag et al., 1995). The components of the media used is highly dependent on the species, however most contain bicarbonate, calcium and macromolecules such as

BSA (bovine serum albumin) (Visconti et al., 1998). Furthermore, the majority of porcine IVF media is supplemented with caffeine (Cheng, 1986; Nagai, 1993; Nagai, 2006; Mattioli et al., 1989) and in some reports ophylline has been included in the media in order to improve boar sperm capacitation rate (Yoshioka et al., 2011). Overall, caffeine is considered the most effective promoter of sperm capacitation, resulting in spontaneous acrosome reaction (Nagai, 1993; Funahashi, 2000; Funahashi et al., 2000) and a significant increase in sperm penetration. However, the use of caffeine supplementation additionally results in a high rate of polyspermy. As an alternative, others have suggested that adenosine supplementation may be used to improve capacitation rate whilst reducing the polyspermic rate associated with caffeine supplementation (Funahashi, 2004; Funahashi, 2000; Funahashi et al., 2000).

It has been reported that boar in vivo in capacitation requires between 90 and 180 minutes after insemination (Mattioli et al., 1996). Therefore, this chapter aimed to optimise the boar sperm capacitation period. Since acrosome reaction is essential for the sperm capacitation process, it is possible that sperm capacitation rate may be correlated with sperm morphological features. This phenomenon is largely under-explored however and therefore work in this chapter additionally aimed to address this gap (Gadea et al., 2005).

4.1.1 Specific aims of this chapter

Specific aim 2a: To test the hypothesis that by decreasing boar sperm concentration, fertility rate is increased, as identified by an increase in cleavage rates.

Specific aim 2b: To test the hypothesis that sperm incubation time is associated with capacitation in the high quality and poor quality boar semen.

Specific aim 2c: To test the hypothesis that supplementation of capacitation media with caffeine or adenosine is associated with higher capacitation rates in the high and poor quality boar semen.

Specific aim 2d: To test the hypothesis that specific morphological parameters in boar sperm are significantly associated with sperm capacitation rate.

Specific aim 2e: To test the hypothesis that sperm capacitation rate is significantly associated with fertilisation potential, as demonstrated by embryo cleavage rates.

4.2 Results

4.2.1 The impact of sperm concentration on fertilisation rate (specific aim 2a)

In this section, IVF was performed using a sperm concentration of either 1×10^6 sperm/ml or 1×10^4 sperm/ml (in this experiment 224 oocytes of Large White were fertilised with Pietran spermatozoa). IVF success was measured in each group by assessing the number of embryos that reached 2 cell, 3 cell, 4 cell, 8 cell, 16 cell, morula stage, early blastocyst and full blastocyst. A Chi square test was performed to test the data for significant differences. Table 4.1 indicate the fertilisation outcomes in each of the two groups. Overall, there was no significant difference between fertilisation outcomes in two groups.

	Sperm concentration				
Embryo outcome	10^6	10^4	Chi square	df	P-value
Development 1 cell to 2 cells	43/152	53/183	0.0183	1	0.892
Development 2 to 4 cells	23/43	34/53	1.119	1	0.290
Development 4 to 8 cells	11/23	16/34	0.0016	1	0.968
Development 8 cell to compacted	10/11	16/16	1.510	1	0.219
Development compacted to morula	9/10	15/16	0.9410	1	0.332
Development morula to blastocyst	0/9	1/15	0.626	1	0.429

Table 4.1. IVF success rates following insemination with 1×10^6 sperm/ml compared to insemination with 1×10^4 sperm/ml. In this experiment, 224 oocytes were used and oocytes were fertilised with Pietran spermatozoa. Chi square test revealed no significant difference between the two between the two groups.

4.2.2 The impact of sperm incubation time on sperm capacitation rate (specific aim 2b)

Capacitation was assessed in semen samples from three high-quality and three poor-quality boars' semen (White Duroc, Pietran and Hampshire), using chlortetracycline (CTC) staining as described in section 2.3, 2.3.1 and 2.3.2.

When CTC staining was performed, three different fluorescence patterns were observed as shown in figure 4.1. Uncapacitated spermatozoa (Pattern A) was identified by bright fluorescence over the entire sperm head and midpiece of the tail; capacitated spermatozoa (pattern B) was identified by presence of fluorescence in the equatorial segment and midpiece of the tail, and a dark band (fluorescence free) in the post acrosome region; or acrosome reacted sperm (pattern C) identified by low fluorescence signal in the sperm head with a positive signal in the equatorial segment and midpiece.
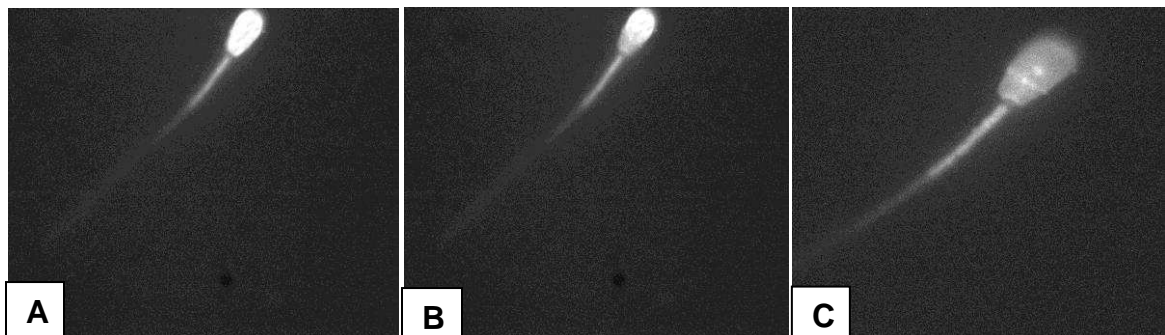
Figure 4.1. CTC staining patterns observed under capacitating conditions. (A) Uncapacitated, acrosome intact sperm, identified by bright fluorescence over the entire sperm head and presence of positive fluorescence in the mid piece of the tail; (B) capacitated, acrosome intact sperm identified by bright fluorescence observed on the equatorial segment and mid piece of the tail. In the post acrosome region fluorescence, free (dark) bands were observed. (C) Acrosome reacted sperm with no fluorescence signal observed on the entire sperm head and positive staining on the equatorial segment and mid piece of the tail. In this experiment three high-quality semen and three poor-quality boar semen were used (two animals were used in each breed)

The percentages of the three different patterns observed throughout the capacitation incubation times in three high-quality and poor-quality semen groups are indicated in table 4.2 (two animals of each breed were used). According to these results, a significant decrease in the rate of pattern A (uncapacitated) was observed in high quality boar sperm group as the incubation time for capacitation proceeded. The percentage of sperm representing pattern A decreased from 54.3±0.5 (mean ± SEM of 6 replicates) at the beginning of the incubation to 38.7± 2.1(n=6), 28.6±3.1 (n=6), 25±2.3 (n=6) and 17±3.5 (n=6) after 60 min,120 min,180 min and 240 min of the incubation respectively. Pattern C (Acrosome-reacted sperm) indicates inverse behaviour compared to pattern A, as the percentage of sperm stained as per pattern C increased from 43.1±0.9 (n=6) at the beginning of the incubation to 45.9±3.7 (n=6), 48.7±3.5 (n=6), 57.5±2.5 (n=6) and 78.5 ±6.1 (n=6) after 60 min, 120 min, 180 min and 240 min of incubation respectively. The percentage of sperm representing pattern B (capacitated) significantly increased from 8.3±0.7 (n=6) at the beginning of the incubation to 25.3±2.6 (n=6) after 60 min of incubation. After that, pattern B increased slightly to 30.1±2.3 (n=6) after 120 min incubation followed by a decrease in the percentage of sperm represented by pattern C to 25.2±2.0 (n=6) and 12.3±3.8 (n=6) after 180 min and 240 min of incubation respectively. The same observation of capacitation patterns indicated for poor semen quality group according to table 4.1.

High quality boar semen				Poor quality boar		
Time (minutes)	A	B	C	A	B	C
0	54.3±0.5	8.3±0.7	34.3±4.9	35.6±14	3.8±0.7	71±4.7
60	38.7±2.1	25.3±2.6	45.9±3.7	12.2±1.8	2.6±0.5	90.0±1.3
120	28.6±3.1	30.1±2.3	48.7±3.5	9.8±1.7	5.5±1.3	91.7±3.8
180	25±2.3	25.2±2.0	57.5±2.5	10.5±0.8	5.8±1.9	88.3±1.2
240	17±3.5	12.3±3.8	78.5±6.1	8.8±3.1	9.7±3.7	90.3±8.7

Table 4.1. The value of three fluorescence patterns during incubation of fertile and sub fertile boar sperm up to 240 minutes. Each value represents the mean of 6 replicates ±Standard error of the mean (SEM), a minimum of 100 spermatozoa were scored in each slide.

In order to determine whether there is any association between incubation time and capacitation rate in both high-quality and poor-quality semen group, a Pearson's correlation test was carried out. According to this statistical test, there was no correlation between incubation time and total capacitation rate in high-quality boar sperm (r = 0.157, p = 0.171), whereas a moderate positive correlation between incubation time and capacitation rate was observed in the poor-quality semen group (r = 0.434, p<0.0001)

4.2.3 Comparison of three different capacitation media for in vitro fertilisation (specific aim 2c)

In this section three different fertilisation media (media 1: capacitation media, media2: capacitation media supplemented with caffeine and media3: capacitation media supplemented with adenosine) were compared to determine which media is more suitable for inducing optimum capacitation rate in both high and poor quality semen groups. In order to identify where overall differences occurred, the capacitation rate in the three-

defined media, was compared for statistical significance using a t-test (results shown in

tables 4.4). Table 4.2 indicates the capacitation rate of high quality and poor quality semen

groups in the three-different media (media1, media 2 and media 3) tested. In high quality

semen group, the Capacitation rate was significantly higher in capacitation media (media1)

compared to the capacitation rate in media supplemented with caffeine (media2) or

adenosine (media3). However, the capacitation rate was significantly higher in media

supplemented with caffeine (media2) compare to adenosine (media3). In poor quality

semen group, the capacitation rate was significantly higher in media supplemented with

adenosine (media3) compare o media supplemented with caffeine (media2) (p value:

0.03).

	t-test value between media1 and media2	df between media1 and media2	p-value between media1 and media2	t-test value beween media1 and media3	df between media1 and media3	p-value between media 1 and media3	t-test value media2 ,media3	df media2,3	P-value Media2,3
High-quality semen	2.932	19	0.01	4.98	16	0.006	3.29	13	0.006
Poor-quality semen	0.640	17	0.531	3.10	11	0.010	2.49	13	0.030

4.2. High-quality and poor-quality boar spermatozoa capacitation rate was compared in three different media: capacitation media (media1), capacitation media with caffeine (media2) and capacitation media with adenosine (media3). T-test was performed to compare capacitation rate in three defined media. According to t-test in high quality boar spermatozoa, the capacitation rate is significantly higher in Capacitation media(media1), and also capacitation rate is significantly higher in capacitation media supplemented with caffeine (media2) than capacitation media supplemented with adenosine. In poor- quality semen group, the mean number of capacitated spermatozoa increase significantly in capacitation media supplemented with adenosine (media3)

This study results indicate that there is a significant difference in proportion of capacitated spermatozoa in high quality boar semen group and poor quality semen group in two tested media. Table 4.3 indicated these differences based on Mann Whitney U test. According to this table the number of capacitated spermatozoa in capacitation media (media1) and in capacitation media with caffeine supplement (media2) is significantly higher in fertile group (p <0.001), while no significant difference observed between the amounts of capacitated spermatozoa in capacitation media with adenosine supplement in both groups (p= 0. 4832).

	High-quality semen	Poor-quality semen	Mann Whitney U test value	df	P value
Mean Capacitated spermatozoa in media 1	28.9±1.4	6.1±0.9	13.345	17	<0.001
Mean Capacitated spermatozoa in media 2	23.8±1.0	7.1±1.3	10.351	19	<0.001
Mean of capacitated spermatozoa in media 3	15.7±2.2	13.1±2.1	0.851	17	0.4832

Table 4.3. A comparison of capacitation rate in fertile and sub fertile spermatozoa in three different media: Capacitation: media 1, capacitation supplemented with caffeine: media 2 and capacitation supplemented with adenosine: media3

4.2.4 Correlation between boar spermatozoa capacitation rate and fertilisation rate (specific aim 2d)

In order to determine the effects of different media supplements on IVF success, capacitation and fertilisation rates were assessed when using high quality boar sperm in media 2 and media 3. 145 oocytes were fertilised in media supplemented with caffeine (media2) and 103 oocytes were fertilised in adenosine (media3) supplemented with adenosine as described in the materials and methods sections 2.3, 2.3.1. Results were tested for statistical significance using an independent samples t-test, as shown in table 4.8. No significant difference was observed in the fertilisation rate between the media tested. In this section, poor quality boar spermatozoa were not assessed as the IVF in our laboratory was only performed with high quality boar spermatozoa in order to achieve higher fertilisation rates. Furthermore, media 1 was not assessed as our IVF protocol stipulates use of supplementation.

Embryo outcomes	The number of fertilised embryos in media 2	The number of fertilised embryos in media 3	Chi-square value	df	Chi-square P value
From 1-2 cell	38/145	39/103	3.823	1	0.053
From cells-4 cells	22/38	19/39	0.651	1	0.420
From 4 -8 cells	18/22	13/19	0.992	1	0.319
From 8 cells-16 cells	15/18	7/13	3.186	1	0.074
Compacted	13/15	4/7	2.431	1	0.119
Morula	12/13	4/4	0.643	1	0.423
Early blastocyst	4/12	2/4	3.111	1	0.551
Blastocyst	1/4	1/2	0.375	1	0.540

Table 4.4. Fertilisation outcomes using two different fertilisation media: capacitation media supplemented with caffeine or adenosine. This experiment was carried out using 145 incubated embryos in the capacitation media with caffeine and 103 incubated embryos in the capacitation media with adenosine. A chi-square test revealed no significant difference between fertilisations rate in the two groups (P value>0.05).

4.2.5 Correlation between boar spermatozoa capacitation and sperm morphology (specific aim 2d)

In order to test for an association between sperm morphology and capacitation potential,

sperm morphology was assessed in three high quality boars and three poor quality boars.

This experiment was repeated three times for each breed and 150 sperm was analysed per

slides. In total 1350 sperm were evaluated in each group of high quality and poor quality

semen, as described in section 2.3 and 2.3.1. As determined in section 4.2.2, the optimum

incubation time for capacitation was 120 minutes and 60 minutes for high quality and poor quality boars respectively. Analysis using a Chi squared test for significance found that sperm of poor quality boars possessed a significantly lower percentage of normal spermatozoa and a significantly higher percentage of sperm with acrosome, tail, head shape and midpiece abnormalities. However, no difference was found in cytoplasmic retention between the two groups. The most common spermatozoa abnormality among sperm from high quality boars was acrosome abnormality with 12.4%, whereas in poor quality boars the most common abnormality was coiled and folded tail with 22.5%. This is illustrated in table 4.

An independent samples t-test was carried out in order to analyse the data obtained for statistical significance. Results showed that the sperm of the high quality boar semen group possessed a significantly higher capacitation rate (58.6%) than that of the poor quality semen group (23.5%, p = <0.05). This is shown in table 4.5 and figure 4.2.

Sperm Feature	Percentage		Chi-square value	df	Chi-square P value
	High quality boar sperm	Poor quality boar sperm			
Normal	58.6%	23.5%	25.456	1	<0.001
Percentage of Acrosome abnormalities	12.4%	21%	2.658	1	0.103
Percentage of Coiled/folded tail	3.0%	22.5%	17.091	1	<0.001
Percentage of Retained Cytoplasmic	12%	10.0%	0.204	1	0.651
Percentage of Head shape/size	11%	18.0%	1.976	1	0.160
Percentage of Midpiece abnormality	3%	5%	0.521	1	0.470

Table 4.5. Boar spermatozoa morphology in high quality and poor quality boars. Three breeds were analysed in each group and the experiment was repeated three times for each breed in both groups. Spermatozoa characteristics between the high and poor quality groups were compared using a classical chi-squared test; differences were considered as significant for p<0.05.
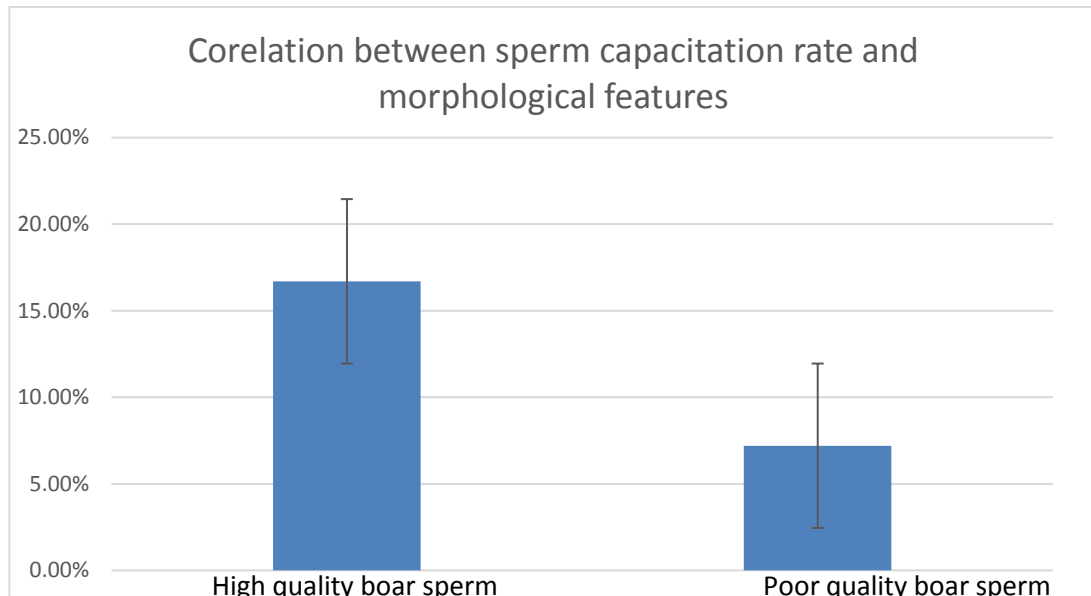
Figure 4.2. Sperm capacitation rate in high and poor quality f boars. Sperm of high quality boars possessed a significantly higher capacitation rate than sperm of poor quality boars.

Following morphological analysis, CTC staining was performed in order to determine whether a correlation exists between spermatozoa morphology and capacitation rate. A non-parametric spearman's correlation test was carried out in order to test for a statistically significant relationship. Results indicate a strong positive correlation between sperm morphological features and capacitation rate, r_S (14) =0.735, P<0.0005. As shown in figure 4.3, a high percentage of normal spermatozoa morphological features are correlated with a high capacitation rate.
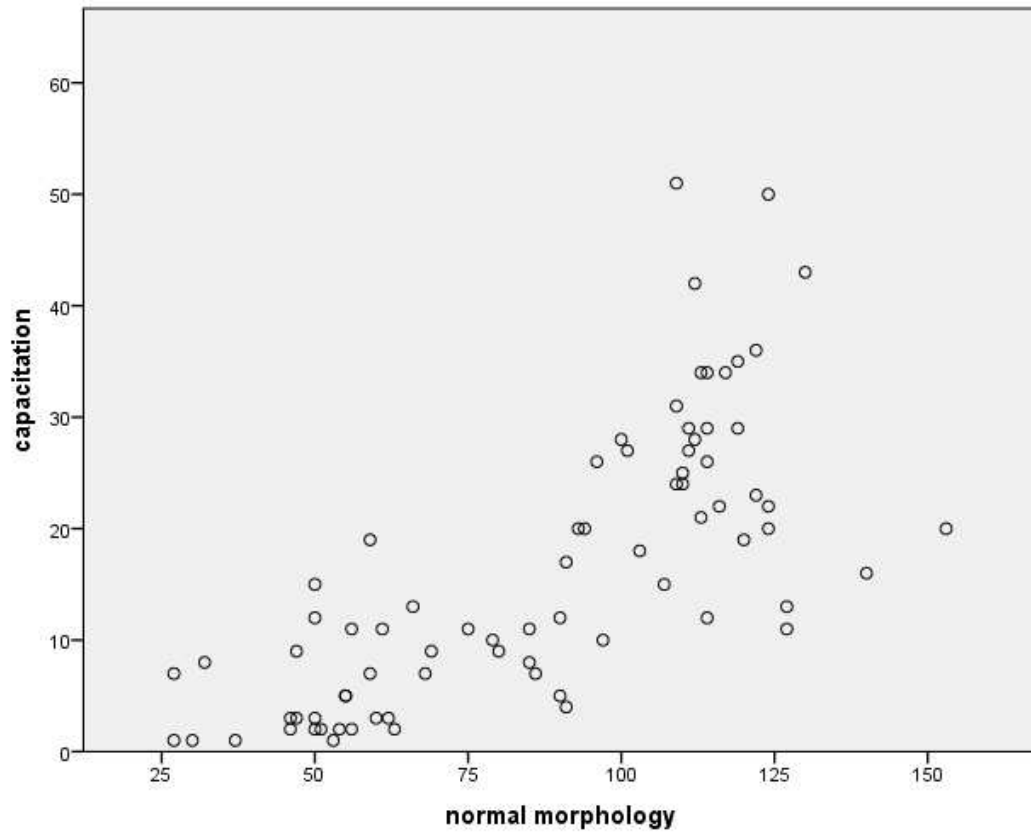
Figure 4.3. Correlation between % morphologically normal sperm and capacitation rate. An increase in the proportion of normal sperm morphological features is strongly positively associated with increased capacitation rate.

4.3 Discussion

4.3.1 Impact of sperm concentration on fertilisation rate (specific aim 2a)

Previously published data has shown that use of a high concentration of boar spermatozoa in IVF treatment results in high penetration rate and an increased incidence of polyspermy (Xu et al., 1996). Furthermore, results have shown a clear negative correlation between polyspermy rate and spermatozoa capacitation rate, by increasing sperm polyspermy the capacitation rate will decrease. As such, it has been suggested that a decrease in the number of spermatozoa used for in vitro fertilisation could reduce the incidence of polyspermy and in turn, increase penetration rate (Gil et al., 2008). In this study, therefore, two different concentrations of boar semen were used in IVF procedures (1×10^6 and 1×10^4 sperm/ml) in order to determine the optimum sperm concentration for fertilisation. The boar sperm motility o each breed was assessed before fertilisation and he average of motility was between 70% and 80 %. This experiment was repeated six times with two different breeds. Our results showed that there was no significant difference in fertilisation rate between the two groups assessed. One of the reasons for this unexpected finding may be explained by the fact that we used cleavage rate as an indicator of fertilisation. Since the porcine oocyte is lipid rich and therefore extremely dark, it was not possible to visualise and assess the rates of pro-nuclei formation as an indicator of successful fertilisation. Thus, it is possible that in some instances fertilisation did occur, however cleavage was not observed. This may lead to an inaccurate estimation of fertilisation rates, which may have affected the interpretation of our data. Alternatively, it is possible that our results can be explained by poor semen samples, since in most cases, the semen used had been collected five to seven days prior to use. In all IVF cycles, fresh semen (as oppose to frozen and thawed semen) were used for fertilisation, therefore it is possible that

during those 5 to seven days from collection to use, sperm quality had significantly

reduced resulting in impaired fertilisation potential. In this instance, we might expect that

varying concentrations would have no effect on fertilisation potential as previously

confirmed by Xu and colleagues (Xu et al., 1998). Finally, it is possible that we did not see

an increase in fertilisation rates with reduced sperm concentration due to the fact that

although a reduction in sperm concentration is expected to reduce polyspermy rates, a

lower number of sperm per oocyte ratio is incurred. It is possible therefore that there

simply were not enough sperm present in the lower concentration group.

4.3.2 The impact of incubation time on sperm capacitation rate (specific aim 2b).

Results from this chapter showed that, as expected, in response to increased incubation

time, the proportion of uncapacitated sperm (pattern A, figure 4.1) progressively decreased,

while the proportion of capacitated sperm and acrosome reacted sperm (patterns B and C

respectively, figure 4.1) progressively increased with incubation time in both high and poor

quality boars. These observations are in accordance with previous findings from Mattioli and

colleagues (Mattioli et al., 1996).

Previous reports have found that spermatozoa capacitation timing is different among

different species in vitro; for example, in humans the capacitation process takes 1 hour while

in the mouse this process is completed in 2 hours. Furthermore, others have noted that in

boars, capacitation is completed in 2 hours (Bavister 1969; Iritani, 1977; Hunter et al., 1974).

This is in agreement with the findings from this study, in which the capacitation process was

seen to be completed within 120 minutes in fertile boars. In fact, our results suggest that the

majority of sperm have undergone capacitation within 60 minutes in high quality boar semen

groups. This is followed by a small but significant increase in the number of capacitated

sperm between 60 and 120 minutes. Following this time, no significant difference in the

proportion of capacitated sperm was observed between 120 and 180 minutes and

interestingly a significant decrease in the proportion of capacitated sperm was observed between 180 and 240 minutes in the sperm of high quality boars. Also, pattern C (acrosome-reacted sperm) proportion increased constantly over the period of incubation from 60 minutes to 240 minutes. It can be argued that sperm is expected to constantly go through the acrosome reaction procedure (which is required to complete the capacitation process). However according to our study, after 240 minutes of incubation, sperm were not able to continue the capacitation procedure. It is possible that this finding can be explained by the presence of sperm with acrosome abnormalities in the ejaculate of high quality boars' semen. In this instance, those sperm with normal acrosome are able to undergo capacitation within the expected time frame, whereas those with abnormal acrosome remain uncapacitated, representing a decline in capacitation at later time points. Alternatively, the decline in capacitation rate after 240 minutes could be an artefact of the experiment, as is is expected that the amount of capacitation should stay constant, or increase over the incubation period rather than begin to decline.

In poor quality boars, our results showed that again, a significant proportion of sperm had undergone capacitation within the first 60 minutes. However, no significant increase in capacitation was observed between 60 and 120 minutes. Interestingly, in contrast to high quality boar semen groups, a significant increase in capacitation was observed between 120 and 180 minutes and 180 and 240 minutes in poor quality boars. It is possible that this may be the result of two separate populations of spermatozoa within the ejaculate of poor quality boar semen: one in which the sperm quality is comparable to that of high quality boar semen and hence undergo capacitation within 60 minutes, and a second in which sperm quality is poor, with high morphological abnormalities and hence capacitation may eventually occur albeit at a much slower rate than those of higher quality. To the best of my knowledge this

is the first study to assess capacitation rate in the sperm of poor quality boars, therefore these findings represent some interesting new insights.

4.3.3 Comparison of three different capacitation media for in vitro fertilisation (specific aim 2c, 2d)

The molecular mechanism of sperm capacitation is still poorly understood. Sperm capacitation may occur spontaneously in vitro in defined media without addition of biological fluids (Visconti et al., 1998), however, previous reports have suggested that various supplements may be added to the fertilisation media in order to facilitate capacitation (Funahashi et al., 2000). The most common examples of such supplements include caffeine and adenosine. In order to investigate the effects of these on the capacitation of sperm from high and poor quality boars' semen, we incubated semen samples from each group with capacitation media alone, capacitation media supplemented with caffeine and capacitation media supplemented with adenosine. Results showed that in both high and poor quality boar semen groups; no significant difference was observed when the fertilisation media was supplemented with caffeine. However, when the media was supplemented with adenosine a differential response was recorded between high and poor quality boar semen groups: While the poor-quality boar semen group showed a significant increase in capacitation in response to adenosine supplementation (when compared to capacitation media alone and capacitation media supplemented with caffeine), the fertile group showed a significant decrease in capacitation in response to adenosine supplementation. Our results are therefore in partial agreement with those reported by others in which it was suggested that the addition of caffeine to IVF media results in an increase sperm motility, induction of capacitation and spontaneous acrosome reaction. (Funahashi et al., 2001). In our own experience, this beneficial effect is specific to poor quality semen samples only. Indeed,

others have also reported boar specific effects of IVF media supplementation on sperm capacitation rates: In a report by Gil and colleagues, while some boars displayed higher capacitation rates in media caffeine supplements, others displayed higher capacitation and fertilisation rates in IVF media without caffeine supplements (Gil et al., 2008). Therefore, it can be concluded that each boar responds differently to caffeine and adenosine supplements. Also, it was reported that when spermatozoa were incubated in the capacitation media with adenosine supplements, they require more time to induce capacitation rate compared to capacitation media with caffeine supplementation (Funahashi et al., 2001). It is possible that spermatozoa incubated in the capacitation media with adenosine supplementation might require additional time to incubate as the number of capacitated spermatozoa in this media declined. According to our results, poor quality boar semen reached capacitation later than high quality boar semen when incubated without supplements, therefore the addition of adenosine supplementation resulted in improved capacitation rates in this group. In another part of this experiment, the fertilisation rate of porcine oocytes in capacitation media with caffeine or adenosine supplementation was determined in order to examine if these results are in accordance with our capacitation findings. It was found that there is no significant difference between fertilisation rates in the capacitation media with caffeine or adenosine supplements, however the IVF experiment in this study was only performed with high quality boar spermatozoa. This study showed no significant difference in the portion of capacitated spermatozoa from high quality semen in capacitation media supplemented with caffeine in comparison to that supplemented with adenosine, therefore it is in accordance with the fertilisation experiment.

4.3.4 Correlation between boar sperm morphological features and capacitation rate (specific aim 2e).

To date, the current literature regarding a correlation between spermatozoa morphology and fertilisation rate remains controversial. While some studies argue a strong correlation, others report none. For example, Lundin and colleagues report that fertilisation ability is not necessarily impaired in morphologically poor human spermatozoa (Lundin et al., 1997), whereas Enginsu et al. argue the opposite (Enginsu et al., 1991) .Interestingly, evidence shows that ICSI treatments can be significantly improved by injecting morphologically normal spermatozoa, suggesting a positive link between morphology and fertilisation ability, however this conclusion requires further validation by additional clinical studies (Bartoov et al.,, 2003). In the pig breeding industry, spermatozoa motility and morphology evaluation are considered as important tools to guarantee successful fertilisation. In support of this, some studies have found a significant correlation between sperm parameters and farrowing rate or litter size (Waberski, 1994; Xu, 1998; Alm, 2006; Ruiz-Sánchez 2006; Broekhuijse et al., 2011). In addition, Alhouse and colleagues found that there is a negative correlation between spermatozoa cytoplasmic droplet and fertilisation rate in boars (Althouse et al.,1995), and Thundathil group found a similar result in bulls (Thundathil et al., 2001). On the other hand, others have found no correlation between cytoplasmic droplet and litter size and fertilisation rate in boars, and more intriguingly, the percentage of sperm possessing acrosome abnormalities has been positively correlated with fertilisation rate (Quintero-Moreno, 2004). Since current literature is sparse and unclear regarding the influence of boar sperm morphology on fertility potential therefore, the aim of this section was to re-visit this topic, in the interest of shedding further light. Results showed that there is a significant correlation between boar sperm morphology and capacitation rate. Overall, the capacitation rate was significantly higher in morphologically good semen samples (p value: 0.03351). This study showed that

21% of spermatozoa used in the capacitation experiment had an acrosome abnormality, while this proportion in the fertile group was 12.4%. Therefore, it is possible that acrosome abnormalities (the second most common abnormality in sub fertile boars in our study) is negatively associated with capacitation. When spermatozoa undergo the capacitation process, the acrosome reaction is initiated enabling the sperm to acquire the ability to penetrate the zona pellucida. In light of this, it is clear that an increase in the percentage of sperm with normal acrosome is associated with an increase in sperm capacitation. It can be concluded therefore, that acrosome morphology plays an essential role in boar fertilisation success rate. Furthermore, our results demonstrate that capacitation may be considered as a suitable indicator for the determination of boar fertility potential.

4.3.5 Conclusion

In conclusion, this chapter has identified that a decrease in boar sperm concentration did not significantly improve fertilisation outcomes, as previously suggested by other reports. In addition, it was found that the optimum sperm incubation time and IVF media supplementation appears to be specific to the boar semen sample used and our results regarding media supplementations was in accordance with fertilisation rate in pig IVF. Finally, results in this chapter identified that sperm morphology parameters are strongly significantly associated with capacitation. This suggests that sperm morphology can act as a suitable indicator of boar fertility potential.

Future work should focus on investigations into a broader range of sperm concentrations in ejaculates from different boars in order to ascertain whether the optimum sperm concentration for fertilisation is also boar specific. Such work would enable more reliable conclusions to be drawn regarding sperm concentration and fertilisation ability.

5   Specific aim 3: To test the hypothesis that reduced fertility in boars is related to levels of sperm packaging as determined by a range of assays

5.1   Background

The previous chapters of this thesis dealt with assessment of parameters in common usage in human IVF clinics but adapted to a porcine model. There are numerous other factors however that have been associated with reduced fertility in humans and, to a greater or lesser extent, been associated with reduced fertility in pigs. Many of these centres around sperm packaging (Erenpreiss et al., 2006). As outlined in the general introduction, the mammalian sperm head undergoes a series of radical alterations in order to package the genome tightly in the sperm head, a process that must be reversed as the nucleus fertilises the egg and proceeds to syngamy. With suitable adaptations to the porcine system, assays for appropriate measurement of the following should shed light on whether there are further tests that may be used in the future to assess for reductions in boar fertility that might impact on downstream production. Such assays form the basis of this chapter and encompass the following:

   a) The level of chromatin packaging in the sperm. There are several assays for this however a common one that is easily used is the CMA3 stain.

   b) The patterns of nuclear organisation in the sperm, which might be related to infertility in humans (Ioannou et al., 2011).

   c) The proportion of sperm cells that are aneuploid (Griffin et al., 2005). In pigs, unlike in humans, fertility problems in boars may manifest themselves as reduced litter sizes or poor non-return rates, factors that might not even be noticed for individual humans who, in western populations at least, might only have one, two or three children in their lifetime. In the following study, the aim was to take a selection of 5

boars that had, retrospectively, been determined to have fertility issues and compare them to 10 controls.

5.2   Specific aims

With this in mind therefore, this study set out to test the following hypotheses:

Specific aim 3a. That the sperm chromatin packaging was significantly different in the poor quality boar semen group compared to high quality group as assayed by CMA3 staining

Specific aim 3b. That nuclear organisation was significantly altered in the poor quality boar semen group compared to high quality boar semen group using a pan-telomeric probe

Specific aim 3c.   To develop locus specific probes for further investigation of nuclear organization in high and poor quality boar semen group and the determination of aneuploidy levels

5.3   Results

5.3.1   To test the hypothesis that the sperm chromatin packaging was significantly different in the sub-fertile compared to the control group as assayed by CMA3 staining

In order to assess chromatin packaging, chromomycin A3 (CMA3) staining was carried out using sperm from 10 high sperm quality boars and 5 poor sperm quality boars as described in the materials and methods section 2.2.7. Since CMA3 competes with DNA protamine binding sites, a deficiency in protamine within the sperm nucleus is highlighted by a positive (bright yellow) CMA3 stain, as shown in figure 5.1. For each boar, at least 100 images were captured and assessed.
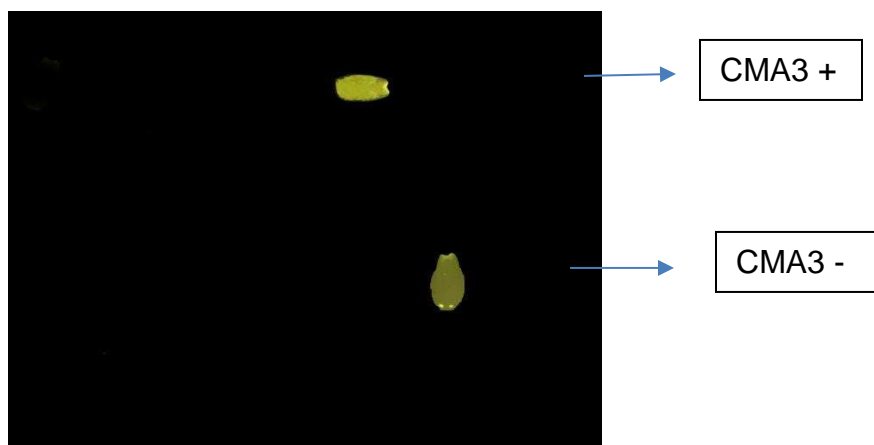
Figure 4.4. Chromomycin A3 (CMA3) staining of boar spermatozoa. Spermatozoa with normal protamine content appear dull, while CMA3 positive or protamine deficient spermatozoa appear as bright yellow (Magnification ×100).

The percentage of sperm nuclei with positive CMA3 staining was calculated, and then all results from each high and poor quality boar semen groups were pooled. Statistical analysis was performed using a Chi-squared test. As shown in figure 5.2, results indicate that the proportion of protamine deficient spermatozoa was highly significantly increased in the sub-fertile group (1.7%) compared to the fertile group (0.5%) (Chi-square value: 7.484, p value: 0.006, df: 1).
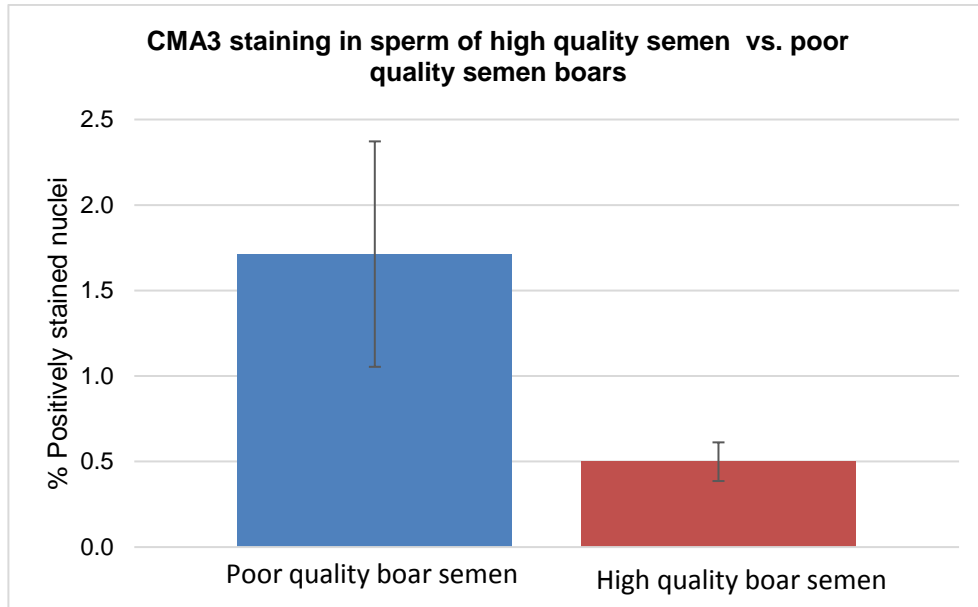
Figure 4.5. Graph to show the proportion of sperm nuclei positively stained by CMA3 in high quality and poor quality boar semen groups. Sub-fertile boars possess a highly significantly higher percentage of positive CMA3 staining (p value=0.006).

5.4   To test the hypothesis that nuclear organization (telomere distribution) in boar spermatozoa is non-random and significantly altered in the poor quality semen group compared to high quality boar semen group (specific aim 3b).

Results from the previous section suggest that chromatin packaging is altered in the sperm of the poor quality semen group, as indicated by a higher proportion of protamine deficient, positive CMA3 stained nuclei. Therefore, in order to investigate this concept further, telomere distribution was assessed in the sperm nuclei of 10 high semen quality boars and 5 poor semen quality boars. Using a telomere specific PNA probe, FISH was performed in sperm samples as described in materials and methods section 2.2.10. A gallery of example images for telomere distribution in control boar spermatozoa can be seen in figure 5.3.
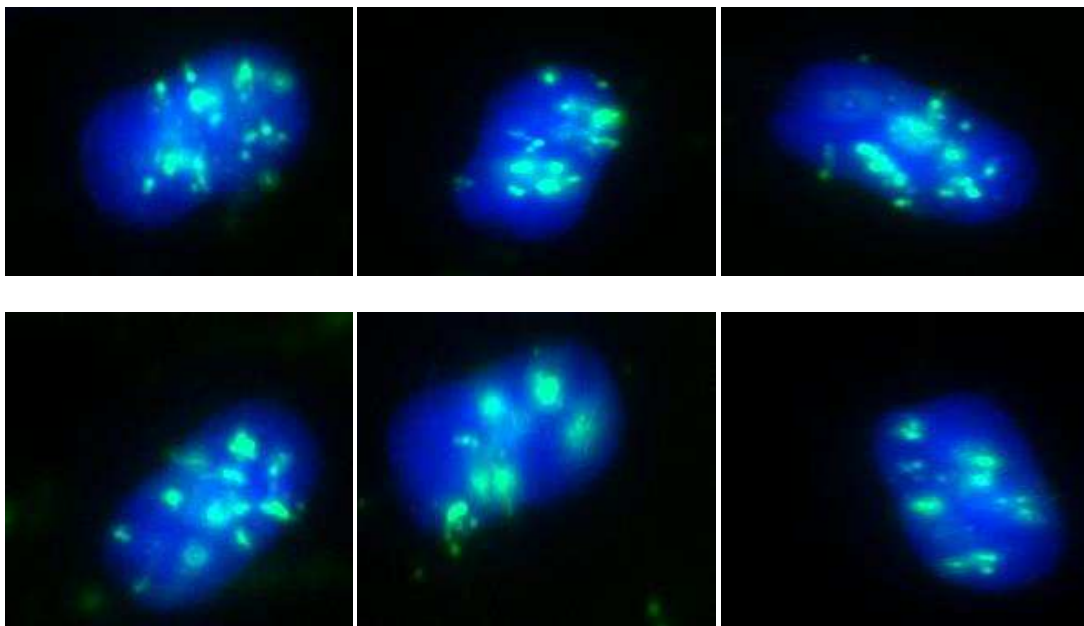
Figure 4.6. Telomere distribution in the sperm nuclei of high quality boar spermatozoa. Images were acquired following FISH experiments using a FITC labelled telomere specific PNA probe (green). The nucleus was counterstained with DAPI (blue).

Figure 5.3 demonstrates that telomeres are positioned throughout the nuclear volume within the sperm head of high quality boar semen. However, as shown in figure 5.4, when normalised for 3D flattening into a 2D image by DAPI density normalisation (as previously described by (Skinner et al., 2009), telomere signals showed a preferential localisation in the interior nuclear volume. Chi-squared analysis revealed that overall telomere distribution was significantly different to random and preferentially located towards the nuclear centre in both control and poor quality boar semen groups (p<0.01)
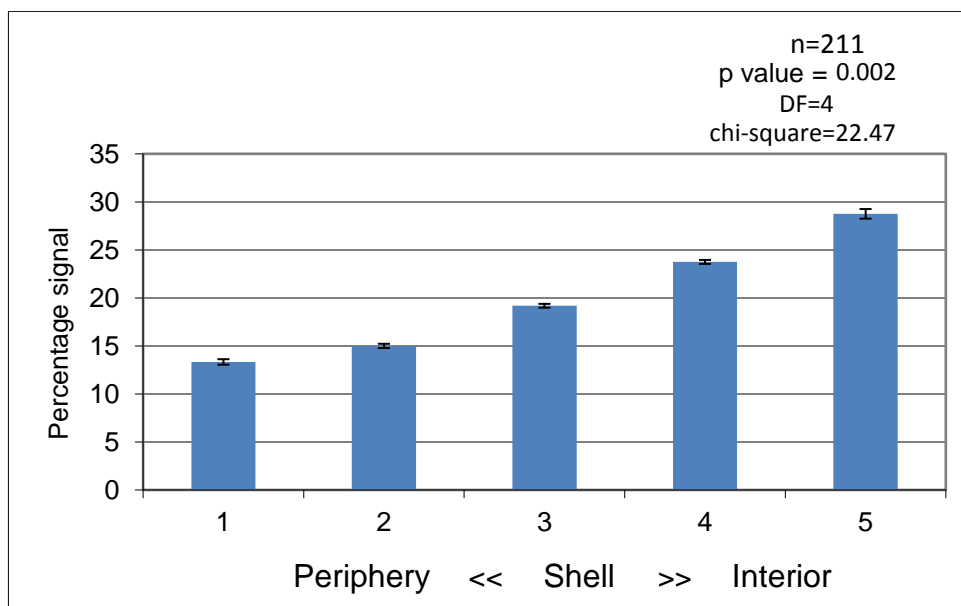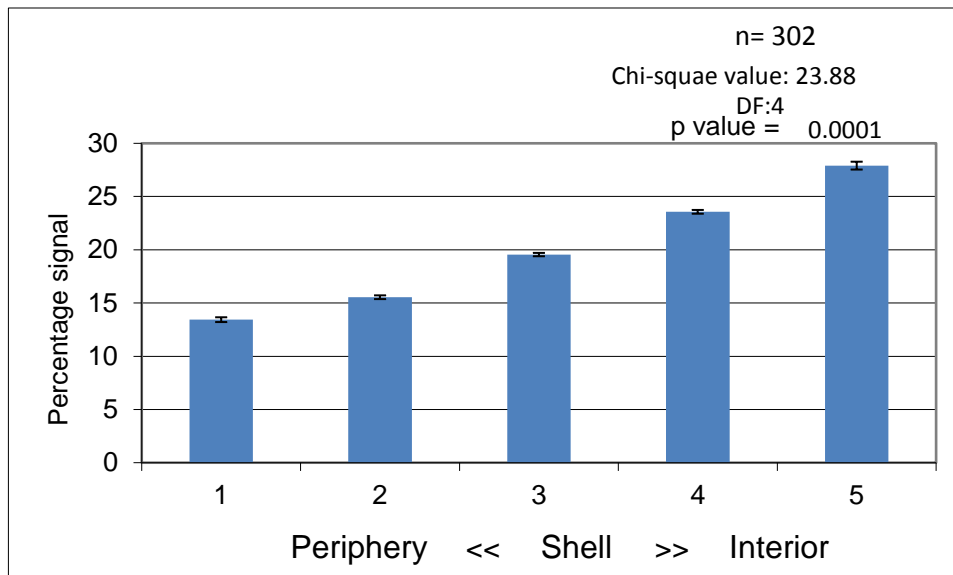
Figure 4.7. Telomere distribution in boar spermatozoa from high quality semen (top) and poor quality semen (bottom. Results indicate that telomeres are preferentially distributed in the nuclear interior in the sperm of high and poor quality boars (p = <0.01).

5.4.1 To develop locus specific probes for further investigation of nuclear organization in high quality and poor quality boar semen groups and the determination of aneuploidy levels (specific aim 3c)

During the course of this study, chromosome specific probes were successfully isolated for chromosomes 1, 2, 3, 6, 7, 10, 11, 12, 14, 16, 17 and 18. Following labelling with red and green fluorochromes a dual colour FISH protocol was optimised in the normal (high quality boar semen) group and poor quality boar semen group. For technical reasons, not clear at the time of writing, the FISH assay was not successful for chromosomes 6, 7 and 17 with the sub-fertile group. The following results for aneuploidy and nuclear organization (chromosome positioning) pertain to the high and poor quality semen groups. Examples of successful FISH experiments are given in figure 5.5
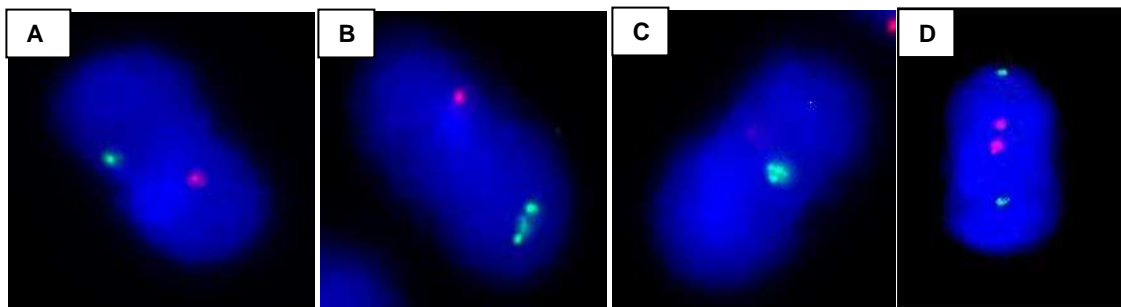
Figure 4.8. The evaluation of boar sperm aneuploidy for autosome chromosomes 2, 18. The green signal represents chromosome 2 and the red signal represents chromosome 18. This figure indicates normal sperm (A), disomy (n+1) (B), nullisomy (2n-2) (C) and diploid (2n) (D).

This experiment was performed on five high semen quality and one poor semen quality boars (two replicates each) and frequencies of numerical aberrations for each of these mentioned chromosomes were assessed in a total of 65000 sperm cells in total (time constraints precluded further analyses). Nullisomic cells (2n-2) were considered an artefact and were not included in to the final calculations as they were not distinguishable from hybridization failure. The estimated aneuploidy rate was calculated by dividing the double

number of disomic sperms by the number of haploid cells, and the rate of diploidy was

calculated by dividing the number of diploid sperms by the number of all examined cells

(haploid and diploid). The average rate of chromosomally unbalanced sperm in high semen

quality and poor semen quality boars was 0.154 % and 0.238%, respectively. Altogether 50

sperm cells had an abnormal number of chromosomes in the high quality boar semen group

and 11 sperm cells had an abnormal number of chromosomes in the poor quality boar

semen group. Among all analysed boar spermatozoa 0.022% diploidy and 0.132 % disomy

levels were identified in the high quality boar semen group and the rate of diploidy and

disomy in the poor quality semen group identified 0.043% and 0.195%, respectively. Table

5.1 demonstrates the disomy, diploidy and estimated aneuploidy rate in both groups of high

and poor quality boar semen groups.

Abnormal number chromosomes	High quality boar semen group	Poor quality boar semen group	Chi^2 value	P value	DF
Disomy	0.132%	0.174%	0.504	0.478	1
Diploidy	0.022%	0.043%	0.797	0.372	1

Table 4.5. Distribution of abnormal number of chromosomes (diploidy and disomy) in high quality boar spermatozoa and poor quality boar spermatozoa. Chi-squared test was carried out in order to test for statistical significance. It was found that there was no significant difference between disomy (0.478) and diploidy rate (P value: 0.372) in high and poor quality boar semen groups.

The Chi-squared test was performed to determine whether there is any significant difference

between diploidy and disomy rate between high and poor quality boar semen groups.

According to the test, no significant difference was observed in either diploidy or disomy

rates in both groups of high and poor quality boars. Table 5.1 indicates the statistical analysis

results. Also, disomy rate in all individual chromosomes were identified as it has been indicated in humans that this varies from chromosome to chromosome (Tempest and Griffin et al., 2005). In order to use the same probes to assess nuclear organisation the approach as described in 2.2.9.1.2 methods and materials section was used. Mean chromosome positions were evaluated for both groups of high and poor semen quality boars by 2D analysis using the DAPI density and volumetric models previously described (outlined in section 2.2.9.1). All autosomic chromosome positioning in the high quality boar semen group is non-random , except chromosome 16, and the poor quality boar semen group indicates non-random positioning in all chromosomes except chromosomes 1,2 and 16 (p = 0.111). Table 5.2 demonstrates the positioning of chromosomes in both groups (with the caveat that a single poor quality boar was examined). A chromosome positioning figure for high semen quality boars has been included in the appendix (figure 8.1 and 8.2). Chromosome positioning for the poor semen quality boar was performed, while this group was only made of one boar (two replicates) chromosome positioning for chromosomes 1, 2, 3, 10, 12, 14, 16 and 18 were performed. Chromosomes in both groups of high and poor quality semen ae located in central region of the sperm head.

High quality boar semen group					Poor quality boar semen group			
Chromosome	Region	Chi square value	df	p-value		Chi square value	df	p-value
1	Central	278.72	4	0.002	Central/Medial	97.64	4	0.325
2	Central/medial	145.28	4	<0.0001	Central	111.40	4	0.064
3	Central	147.8	4	0.0001	Central	234.51	4	<0.0001
6	Central	223.55	4	<0.0001	Did not evaluate for sub-fertile	N/A	4	N/A
7	Central	138.63	4	0.0007	Did not evaluate for sub-fertile	N/A	4	N/A
10	Central	105.79	4	<0.0001	Random	88.64	4	0.184
11	Central	234.81	4	<0.0001	Did not evaluate for sub-fertile	321.4	4	N/A
12	Central	179.78	4	<0.0001	Central	66.16	4	<0.0001
14	Central	88.43	4	0.003	N/A	N/A	4	N/A
16	Random	1.30	4	0.865	Random	5.02	4	0.285
17	Central	100.27	4	0.037	N/A	N/A	4	N/A
18	Central	234.55	4	<0.0001	Central/Medial	36.5	4	<0.0001

Table 4.6. Chromosome positioning in high and poor quality boar groups. Results showed that chromosome positioning is non-random (P <0.05) in both groups, with the exception of chromosome 16 in both group, chromosome 1 and 2 in poo quality semen group which is random (P>0.05).

Taken together therefore the results show little or no evidence of an alteration in sperm aneuploidy or nuclear organization in poor quality semen group vs high quality boars.

5.5 Discussion

5.5.1 To test the hypothesis that reduced fertility in boars is related to levels of sperm packaging at determined by a range of assays (specific aim 3a)

Normal spermatogenesis is necessary for the formation of competent spermatozoa (Iranpour et al., 2014). One important stage in spermatogenesis is the replacement of histone by protamine which is required for the high stability and condensed nature of the chromatin structure in spermatozoa. This sperm condensing and insoluble chemical properties of protamine provides high protection of paternal genome in the reproductive tract of males and females (Brewer et al., 1999). Abnormalities in sperm chromatin packaging and DNA damage are correlated with poor fertility potential (González-Marín et al., 2012). Abnormalities in sperm chromatin packaging can be caused by: disturbances during replacement of protamin with histone, a lack of protamine and/or any disturbances in the process of sperm maturation in epididymis (Kazerooni et al., 2009).It has been reported that sperm DNA integrity is a better indicator of semen quality compared to traditional sperm evaluation techniques (morphology, concentration and motility) (Zini et al., 2001). Most of these studies have been performed in human sperm but very few studies have been done in boar sperm chromatin packaging. In Zini's study, chromatin packaging structure in boar spermatozoa was analysed using CMA3 staining to determine protamine deficiency. Protamine is a specific sperm protein and during decondensation it is replaced histone protein. Therefore, protamine deficiency can impact on decondensation procedure and then fertility potential (Zini et al., 2001). Tarrozi and colleagues (2009) reported that

the interaction between DNA strand breaks and abnormal sperm chromatin packaging can indicate that this abnormality arises in the spermatogenesis pathway involving sperm chromatin packaging and condensation CMA3 in-vivo competes with protamine and is an indirect measure of protamine content such that a high degree of CMA3 staining is possibly an indication of low protamination and sub-fertility in boars. In the present study, the application of CMA3 as an effective indicator of sperm chromatin packaging quality has been investigated as a potentially useful technique in assessing boar fertility

Potential (Tarozzi et al., 2009). It was found that protamine deficiency is significantly higher in the poor quality semen group than in the control (p value <0.05). This result is in accordance with a similar study in human (Iranpour et al., 2014) Protamine deficiency in sub-fertile boars can cause failure in the decondensation process as there is not sufficient protamine to replace the histone protein, therefore it impacts on fertility potential in this group. It can be concluded that CMA3 staining can be considered as an indicator of boar sperm fertility potential, as it is a very convenient technique because it avoids using costly computer assessed sperm analysis (Banaszewska et al., 2015)

5.5.2 To test the hypothesis that nuclear organization (telomere distribution) in boar spermatozoa is non-random and significantly altered in the sub-fertile group compared to control group (specific aim 3b)

Early studies in human genome architecture in the human sperm nucleus indicated a chromosome hairpin-like configuration. Centromeres are located near the centre of nucleus while the telomeres are located in the periphery of the nucleus. It has been reported that telomere distribution in human sperm is non-random and telomeres are mostly located in the periphery of the sperm nucleus (Acloque et al., 2013). In contrast, Hazzouri and colleagues in 2013 did not confirm the location of telomers in human spermatozoa in the peripheral part of nucleus, but their study was quite small and was not reliable (Hazzouri et al., 2013). This is the first study to investigate the telomere distribution in boar spermatozoa in both fertile and sub-fertile groups. According to the present study, telomere distribution in both fertile and sub-fertile boar spermatozoa has a non-random distribution. While this study suggests that telomeres in boar spermatozoa is mostly located in the central part of the sperm nucleus as it is not in accordance with a similar human study (Acloque et al., 2013) .It could be because boar spermatozoa need to go through a decondensation process in order to perform FISH and boar spermatozoa decondense much more in 10mM DTT with 0.2g papain in 0.2M Tris-HCl decondensing agent, therefore the decondensing process itself may cause this alteration of telomere location in boar spermatozoa. Further experiments with different condensation protocols are thus required in future.

5.5.3 To develop locus specific probes for further investigation of nuclear organization in fertile and infertile boars and the determination of aneuploidy levels (specific aim 3c)

Chromosomal imbalances are normally present in the semen of wild type animals due to de novo mutations in the germ line (Saias-Magnan et al.,1999)Dual colour FISH is performed to evaluate chromosomes and to estimate aneuploidy in spermatozoa, therefore for estimating aneuploidy, disomy (presence of an extra chromosome), diploidy (double chromosomal constitution), and nullisomy (absence of one chromosome) while nullisomy cannot be determined by FISH accurately as there is possible that some chromosomes will be loss during fixation therefore in most experiments the number of nullisomy it is quite high which is not reliable, that is why in this experiment for estimating aneuploidy rate, the double number of disomy divided by the rest of normal haploid cells is calculated. The aneuploidy rate was estimated for autosome chromosomes of four high quality boar sperm and one poor quality boar and the aneuploidy rate was quite similar between all animals tested and there was no significant difference between the aneuploidy rate among infertile and sub-fertile boars analysed. As discussed in the introduction, currently only a few authors have used FISH to assess boar spermatozoa copy number of chromosomal abnormalities. Orsztynowicz and colleague indicated that a baseline of 0.105% of chromosomal imbalances (disomy+diploidy) is detectable in fertile boar semen, while in their study aneuploidy rate only for chromosome 1, 10 and Y was estimated (Orsztynowicz, 2011 #516).The present study is the first to estimate aneuploidy for most autosome chromosomes. 32500 high quality boar spermatozoa were analysed in this study and the baseline 0.132% of imbalance chromosome copy number (diploidy+disomy) for the high quality group and the baseline 0.174% of imbalance chromosomes in poor quality group has been found. It has been reported that the frequency of disomy rate in autosome chromosomes in human spermatozoa was 0.15% and 0.26% in sex chromosomes

(Shi et al., 2000) .The disomy rate in boar spermatozoa according to this study is close to the proportion of disomy in human spermatozoa. The present study indicated that the percentage of imbalance chromosome copy number in high and poor quality semen group is not significantly different. This point can be argued that in this study more high quality boar spermatozoa (32500) were analysed for aneuploidy compared to the poor quality boar (4606 sperm cells) and also more chromosomes have been evaluated in the high quality boar semen group as during this study in poor quality semen group some chromosomes such as 6, 7, 11 and 17 did not produce a good signal. Therefore, Dual colour FISH in sub-fertile boars requires more optimisation and this aneuploidy analysis in the sub-fertile group can be considered a preliminary study. It is possible that performing this study with more boars may give a more accurate and reliable comparison between aneuploidy rate in fertile and sub-fertile animals. In human studies, it is reported that sperm DNA with high fragmentation and damage have a higher aneuploidy rate (Carrell et al., 2003). It has been suggested that the rate of aneuploidy in infertile human males will increase and also in human semen with more abnormal sperm parameters indicated higher aneuploidy rate compared to control group (Calogero et al., 2003). In brief, performing FISH on boar spermatozoa is more challenging than human because the decondensation procedure required in animals is different from species to species. For instance, in this experiment papain as decondensing agent was used to obtain better signals, poor quality spermatozoa decondensed significantly better in DTT and papain in Tris HCl compared to DTT in Tris HCl.

The present study suggests that boar spermatozoa are the same as other mammals in having highly organized chromosome territories. The high quality boar spermatozoa indicated non-random positions and these findings are in accordance with

(Foster et al., 2005). This can have considerable implications for the presence of nuclear organization and gene-expression patterns in early embryogenesis (Foster et al., 2005). In the present study, the chromosome positioning in poor quality boar spermatozoa was also mostly considered as non-random with the exception of chromosome 16, which indicated a random localisation. Chromosome positioning of chromosomes 1, 2, 10, 14 and 18 was slightly altered in the poor quality boar semen group compared to high quality boar semen shifting from central to central/medial or vice versa in the poor quality semen group. Some chromosome positioning is not in accordance with (Foster et al., 2005)   findings as they suggested that the position of chromosome 11 is mostly in the peripheral region of nucleus while in this study this chromosome is located in the central region of high quality boar sperm nuclei. The differences between these two studies can be due to different decondensation procedures performed, which may have caused some changes to the chromosome positioning.

Overall these results therefore suggest that the CMA3 stain is worthy of further investigation on a larger group of animals and may ultimately become a useful tool in screening for boar infertility. By and large however FISH studies are still in their infancy and were beset with technical problems that meant that time ran out to perform experiments on more boars, particularly those in the sub-fertile group. Nonetheless, this study has developed the tools for further study that may, in time, reveal whether or not aneuploidy rate and nuclear organization of certain chromosomes is altered in poor quality boars as it is in some sub-fertile humans. In point of fact much of the time was taken getting the assay to work in the first place which proved technically more difficult than in other species such as human, cattle and mouse. Even when it did finally work, the key step was the addition of papain into the swelling buffer and, even then, most of the sub-fertile boars

attempted did not work (and some probes did not work at all). Whether it is coincidence that the poor quality semen group appeared to be disproportionally subject to experimental failure in unclear at this time; if it were the case then perhaps biological reasons that were manifested in the differential CMA3 staining may be at the root cause. Moreover, whether the addition of papain caused a technical artefact that made the telomeres appear as though they were centrally located still warrants further investigation. This is a distinct possibility since other mammalian sperm analysed showed that the telomeres appear to occupy a peripheral nuclear address.

6   Specific aim 4: To investigate the impact of biopsy on embryo morphology and to determine the appropriate time of doing embryo biopsy by using time-lapse

6.1   Background:

Preimplantation genetic diagnosis (PGD) allows embryologists to select healthy, unaffected embryos for transfer (Kirkegaard et al., 2012) in patients carrying a well-defined genetic abnormality. The process of PGD involves the removal of the first and/or second polar body from the oocyte, one or two blastomeres from a cleavage stage embryo or several trophectoderm cells from a blastocyst, followed by subsequent genomic analysis (Harper, 2010). Although there are countless examples of successful outcomes in PGD cases however, the effects of embryo biopsy on developmental potential remains under discussion. An initial study showed that, although removal of up to two blastomeres at 8 cell stage reduced cell numbers in both the trophectoderm and inner cell mass of the subsequent blastocyst, no poor effect on embryo development was observed {Hardy, 1990 #1645}. However, others have shown that embryo biopsy resulted in an increased frequency of blastocyst contraction and expansion movements (Ugajin et al.,2010), delayed and abnormal hatching, delayed development (Malte1989; Cohen;Schmoll2003;Duncan, 2009;Tarin et al., 1992) and reduced embryo quality (Cohen et al., 2007) . Moreover, the number of cells removed was correlated with developmental potential (Goossens 2008; De Vos et al., 2009). In recent years, the introduction of time-lapse incubators designed to allow continuous, non-invasive monitoring of embryo development (Cruz et al., 2011) has enabled a more comprehensive investigation of the effects of embryo biopsy on developmental potential. To the best of our knowledge however, only one study has attempted to do so thus far. Results indicated that the duration of the developmental stage in which biopsy was performed was considerably delayed in biopsied embryos compared with the control group.

Nonetheless, the duration of compaction, morula and early blastocyst stage was identical in both groups. The authors concluded therefore, that in line with previous results from studies that did not utilise time-lapse monitoring, PGD embryos experience developmental delay in comparison to the control group. Interestingly however, the duration of the blastocyst stage was found to be shorter in the biopsied group, resulting in hatching at almost identical timing among biopsied and non-biopsied embryos (Kirkegaard et al., 2012). In another part of this study, time-lapse parameters impact on predicting blastocyst quality is investigated. There are several studies that have been done in this field so far, however results are not constant. It has been found that blastocyst quality can be determined based on embryo morphology on the first 48 hours of culture. Wong and colleagues used time-lapse parameters to identify blastocyst quality for the first time, concluding that the duration of first cytokinesis, duration of 2 cell and 3 cell stage were important predictors (Wong et al., 2010). However, other similar studies reported that only duration of 3 cell can be considered a good predictor of blastocyst quality (Hashimoto 2013; Krikegaard et al., 2013), therefore further research is required in this field. Although this study represents some insightful new findings, further investigation is required in order to obtain accurate interpretations. In this study, only 52 biopsied and 52 control embryos were included; therefore, the aim of this chapter was to asses more embryos in a bid to shed further light on this subject. More specifically, the aims of this chapter were as follows**:**

6.1.1 Specific aims of this chapter

Specific aim 4a: To investigate the impact of embryo biopsy on the timing and duration of defined developmental stages.

Specific aim 4b: To compare treatment outcome in patients that opted for embryo biopsy at the cleavage stage with that of patients that opted for embryo biopsy at the blastocyst stage.

6.2 Results

6.2.1 To investigate the impact of biopsy on embryo morphology and to determine the appropriate time of doing embryo biopsy by using time-lapse (specific aim 4a)

In this section the impact of biopsy on embryo morphology was investigated using the Embryoscope time-lapse device. According to the methods outlined in sections 2.1, 2.1.1 and 2.1.2, 612 cleavage stage biopsied human embryos produced via ICSI treatments were analysed and compared to 220 blastocyst stage biopsied embryos as a control via ICSI treatment. Unfortunately, it was impossible to obtain information on non-biopsied embryos from the clinic. Embryos were incubated in the Embryoscope device following disappearance of 2PN (pronuclear) and were annotated based on standard annotation policy at the Bridge Fertility Centre, London. Embryo viewer software was used to annotate embryos as described in the Materials and Methods in sections 2.1.2.

Images below indicate development of an embryo at some of the key stages in development.
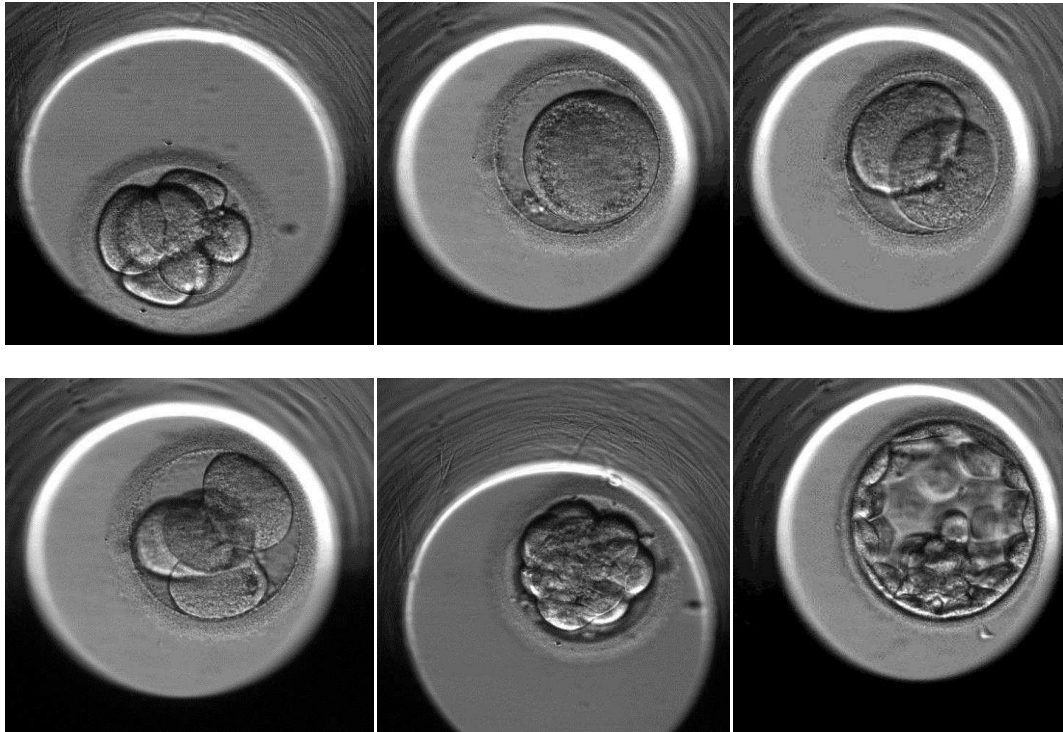
Figure 6.1. 650 embryos were annotated using the Embryoscope time-lapse device. Annotation was performed according to standard morphological makers described in section 2.1.1. (A) represents disappearance of pronuclei, (B) shows the first mitotic division (t2), (C) represents the third mitotic division (t3), (D) represents the 7th division (t8), (E) shows the morula stage of development and (F) the full blastocyst.

Developmental potential of 460 cleavage stage embryos biopsied for PGD at the 8 cell stage was compared to 220 embryos biopsied at the blastocyst stage according to the criteria outlined in table 6.1. Using a Mann-Whitney U test for significance, results showed that although there was a significant delay in the time taken to reach the 2 cell stage between the two groups (p = 0.001), no significant difference was observed in any other time points prior to the 8 cell stage on day 3 (from t2-t9); the time at which biopsy was performed in the cleavage stage biopsy group. Furthermore, results showed that embryos biopsied at the 8 cell stage reached the 9 cell stage (t9) highly significantly later than embryos in the blastocyst biopsy group (p = 0.001). Following the 9 cell stage, the time taken to reach morula stage was longer in the cleavage stage biopsied embryos than the blastocyst stage

biopsy group, however this difference, although close to confidence at the 95% level, was not significantly different between the two groups (p = 0.06). Interestingly, the time taken to reach the start of blastulation (t_{sb}), the full blastocyst stage (t_b) and hatching stage (t_{hb}) was highly significantly shorter in the embryos biopsied at cleavage stage than in the embryos biopsied at blastocyst stage (p = ≤0.001). These results are summarised in figure 6.2 and table 6.1

Control (blastocyst biopsy)				Cleavage stage biopsy			
Embryo stages	n	Time (h) since fertilisation	n	Time(h) since fertilisation	Mann Whitney U test value	df	P value
2- cell stage	220	27.84 ±0.26	612	29.49±0.25	2.40	306	0.017
3-cell stage	208	37.7±0.30	580	38.5±0.28	1.3	175	0.197
4-cell stage	204	39.9±0.60	574	38.96±0.32	3.28	259	0.055
5-cell stage	185	48.7±0.80	524	49.8±0.35	0.130	170	0.900
6-cell stage	184	53.35±0.93	490	53.8±0.50	0.215	242	0.722
7-cell stage	164	58.7±0.90	478	57.43±0.53	0.655	221	0.510
8-cell stage	158	64.1±1.1	460	63.78±0.70	0.030	265	0.976
9- cell stage	125	75.75±1.21	420	79.32±0.9	1.65	242	0.0003
Morula	118	99.33±1.1	368	99.81±0.7	0.420	227	0.675
Start of blastulation	104	108.97±1.2	220	101.63±0.81	5.01	191	<0.001
Blastocyst	96	116.3±1.2	158	106.44±1.02	5.31	182	<0.001
Hatching blastocyst	30	128.5±2.3	40	118.2±1.71	3.71	59	0.0005

Table 6.1. Timing of various developmental stages among embryos biopsied at cleavage or blastocyst stage. Data are expressed as average values with 95% CI. Differences between the two groups were tested for significance using a Mann-Whitney U test.
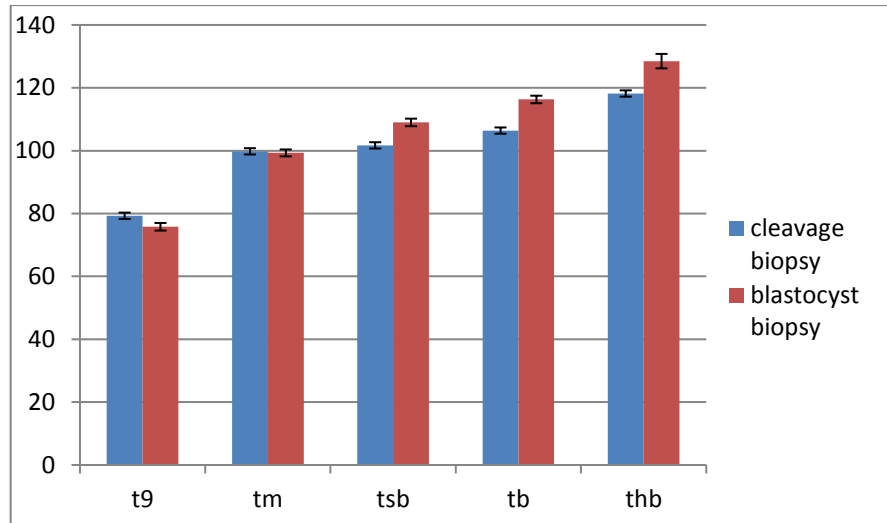
Figure 6.2. Time points of various embryonic stages after biopsy was performed at cleavage stage (blue) or blastocyst stage (red). A highly significant difference was observed between t9 (p = 0.001), start of blastulation (p = <0.001), full blastocyst (p = <0.001) and hatching stages (p = 0.001) between the two groups.

As shown in table 6.2, the duration of each embryonic stage was additionally assessed. Results showed that the duration of the 2- cell stage is significantly shorter in the blastocyst biopsy group. However, after this time, no differences were observed between the two groups until the 8 cell stage. Following the 8 cell stage, although no significant difference was observed between the two groups in the duration of 9 cell stage (t9-t8), morula stage and full blastocyst stage, the duration of the start of blastulation and hatching stages lasted significantly shorter in the cleavage stage biopsy group compared to the blastocyst biopsy group (figure 6.3 and table 6.2).

Blastocyst stage biopsy (control)			Cleavage stage biopsy				
Embryonic stages	n	Time(h) since fertilisation	n	Time(h) since fertilisation	Mann Whiney U test	df	P value
$t_3 - t_2$	220	10.6±0.41	612		3.82	164	0.0002
$t_4 - t_3$	208	9.12±0.41	580	8.42±0.21	4.88	217	<0.001
$t_5 - t_4$	204	11.2±0.62	574	10.3±0.5	1.15	262	0.250
$t_6 - t_5$	185	3.8±0.50	524	4.4±0.3	1.20	212	0.230
$t_7 - t_6$	184	4.5±0.50	490	4.8±0.4	0.450	227	0.655
$t_8 - t_7$	164	6.4±0.73	478	5.9±0.33	0.570	144	0.567
$t_9 - t_8$	158	11.3±0.73	460	11.7±0.50	0.495	187	0.621
$t_m - t_9$	106	17.7±0.71	274	11.7±0.6	6.547	223	<0.001
$t_{sb} - t_m$	101	10.9±0.62	148	4.90±0.31	8.70	148	<0.001
$t_b - t_{sb}$	104	7.7±0.5	220	4.7±0.32	5.50	155	<0.001
$t_{hb} - t_b$	27	10.82±1.1	40	8.54 ±0.60	1.980	45	0.0548

Table 6.2. Duration of each developmental stage in embryos biopsied at cleavage or blastocyst stage. The data shown is demonstrated as average duration with standard error. A Mann-Whitney U test was used to test for statistical significance in any differences between the two groups.
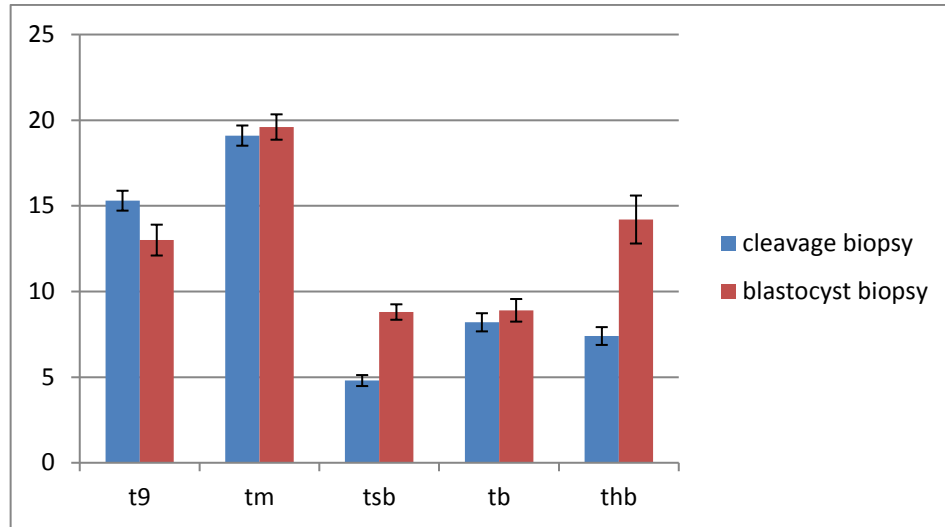
Figure 6.3. The duration of embryonic stages after performing biopsy in cleavage stage (blue) and blastocyst stage (red) biopsy. No significant differences were observed in the duration of the developmental stages assessed until the start of blastulation (t_{sb}-t_m), which was significantly shorter in cleavage stage biopsied embryos (p = <0.001). Although no difference was observed in the duration of the full blastocyst stage (t_b-t_{sb}), hatching was significantly shorter in cleavage stage biopsied embryos compared to blastocyst stage biopsied embryos (p = <0.001).

6.2.2 To compare blastocyst and hatching blastocyst rate and treatment outcome in patients that opted for embryo biopsy at the cleavage stage with that of patients that opted for embryo biopsy at the blastocyst stage (specific aim 3b).

In this section a comparison study has been done between cleavage stage biopsy and blastocyst stage biopsy. Blastocyst and hatching blastocyst rate was evaluated in two groups of cleavage and blastocyst stage biopsy. In this study, 158 cleavage biopsied embryos reached blastocyst stage and only 40 of them developed to hatching blastocyst (25.3%) and 87 of blastocyst stage biopsied embryos reached blastocyst and 25 (28.7%) of them reached hatching. Results showed that there is no significant difference between hatching blastocyst rate in cleavage stage biopsy and blastocyst stage biopsy group. Table 6.3 illustrates these results.

Blastocyst biopsy							Cleavage stage
Embryonic stage	n	Percentage	n	Percentage	Chi-squared value	df	P value
Hatching blastocyst	25/87	28.7%	40/158	25.3%	0.337	1	0.562

Table 6.3. Chi-squared test for significance indicates no difference in the proportion of embryos reaching hatching blastocyst stage between blastocyst stage biopsied embryos and cleavage stage biopsied embryos.

Treatment outcome following embryo biopsy was assessed by comparing implantation rate (as confirmed by a positive biochemical pregnancy test) and live birth rate in couples that opted for embryo biopsy at either the cleavage stage of development or the blastocyst stage. A Chi-squared test for significance found no difference in the implantation rates between the two groups (p = 0.710). Furthermore, no significant difference was observed in live birth rates between the cleavage stage biopsy group and blastocyst stage biopsy group (p=0.695). However, a higher proportion of embryos biopsied at the blastocyst stage were frozen (20%) compared to 5.9% the cleavage stage biopsy group and thus no pregnancy outcome is available for a greater proportion of blastocyst stage biopsied embryos. Table 6.4 and figure 6.5 demonstrate the patient's outcomes in two groups of cleavage and blastocyst stage biopsy.

Patient's outcome	Cleavage stage biopsied embryos%	Blastocyst stage biopsied embryos%	Chi-squared value	df	P value
Live birth	21%	23.3%	0.153	1	0.695
Biochemical pregnancy	2.6%	3.3%	0.086	1	0.770
Miscarriage	12.2%	6.7%	1.768	1	0.184
No normal embryo	11.3%	10%	0.089	1	0.766
No pregnancy	47%	36.7%	2.18	1	0.140
Frozen embryos	5.9%	20%	8.818	1	0.003
Implantation rate	35.8%	33.3%	0.138	1	0.710

Table 6.4. Patient outcomes regarding the percentage of live birth, biochemical pregnancy, miscarriage, abnormal embryos and frozen embryos in cleavage stage biopsied and blastocyst biopsied embryos.
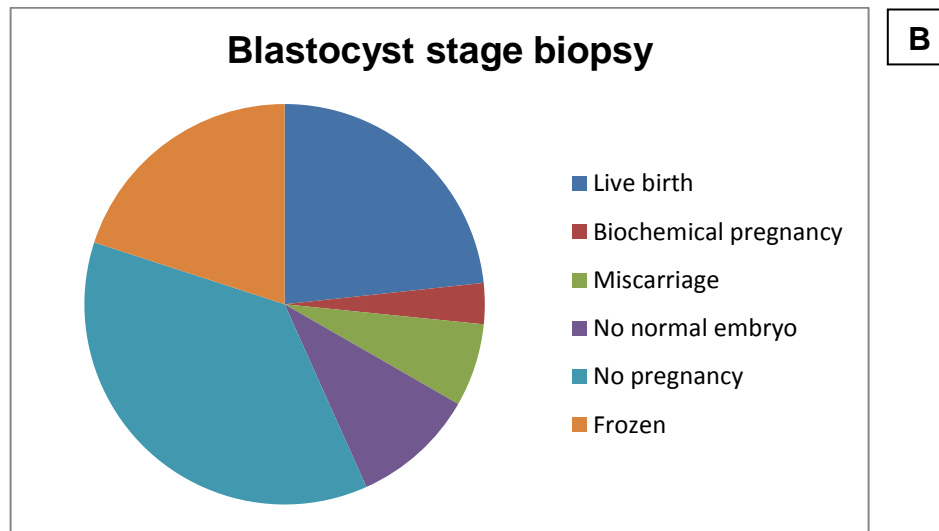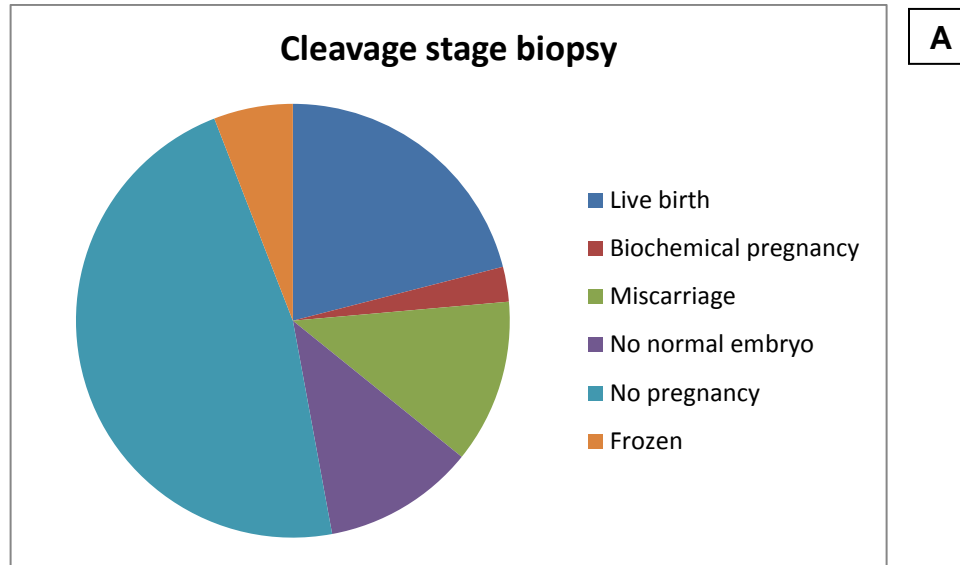
Figure 6.4. Patient outcomes regarding the percentage of live birth, biochemical pregnancy, miscarriage, abnormal embryos, no pregnancy and frozen embryos in cleavage stage biopsy group (A) and blastocyst stage biopsy group (B).

6.3 Discussion

6.3.1 The impact of biopsy on embryo morphology (time-lapse study)

Time-lapse monitoring has provided a detailed and dynamic analysis of the development of human pre-implantation embryos. In this study, an Embryoscope time-lapse device was used to compare the timings and duration of specific developmental events following biopsy at cleavage stage (8 cell stage on day 3) and blastocyst stage (day 5). Results showed that, following biopsy at the 8 cell stage, the time taken to reach the 9 cell stage was significantly longer in cleavage stage biopsied embryos. Interestingly however, the duration of the start of blastulation, and hatching blastocyst was significantly shorter in day 3 biopsied embryos compared to the blastocyst stage biopsy group. This shorter duration period at each of these stages resulted in cleavage stage biopsied embryos reaching subsequent developmental stages significantly earlier than those embryos that were not biopsied; that is, the emergence of the full blastocyst and the time of hatching was significantly earlier in cleavage stage biopsied embryos. In contrast with our findings, a study by Duncan et al observed a developmental delay in mouse embryos that underwent biopsy compared to non-biopsied embryos (Duncan et al., 2009). Similarly, Kirkgaard and colleagues showed that the duration of the stage in which embryos were biopsied was longer than that of the control group, resulting in biopsied embryos reaching subsequent embryonic stages up until hatching considerably later than non-biopsied control embryos (Kirkegaard et al., 2012) Furthermore, we utilised the Embryoscope® time-lapse device in our study, which captures every 15 minutes. It is possible that blastocyst expansion may occur within this time frame and therefore was not accurately captured in the control group, leading to a significantly shorter recorded hatching time point in the cleavage stage biopsied group compared to the blastocyst stage biopsy group. In a previous study, the impact of blastomere removal on the process of hatching has been studied in mice via time-lapse monitoring (Duncan 2009;

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Ugajin et al., 2010) and according to these studies, the time to the end of the hatching stage was significantly longer in the biopsied group, therefore authors confirmed that removal of a blastomere on day 3 negatively impacted the hatching process. It can be discussed that, there was some limitations with this study as the control group for this study was blastocyst stage biopsy group, therefore in this group biopsy was performed at full blastocyst stage and maybe this causes the hatching blastocyst duration in this group to be significantly longer than the cleavage stage biopsy group. Furthermore, this study did not record the expanded blastocyst in all of the cleavage stage biopsy group. Some embryos reached hatching stage immediately after full blastocyst stage and therefore, in some cases full blastocyst stage and hatching was not recorded. Alternatively, it is possible that the process of zona breaching in order to biopsy at the 8 cell stage negates the requirement for blastocyst expansion and zona thinning in order to facilitate hatching. In this instance, the time taken for the embryo to hatch would be reduced, as shown in our results. Indeed, evidence from previous studies has shown that blastocyst expansion and zona thinning was only observed in non-biopsied embryos and not among biopsied embryos. Instead biopsied embryos hatched through the artificial whole in a thicker zona generated in the process of biopsy (Kirkegaard, 2012, Malter, 1989; Montag, 2000; Sathananthan et al., 2003). Although reports have suggested that cleavage stage biopsy provides better sample material in comparison to polar body biopsy, our own results and those of several other investigators have shown that the process of cleavage stage biopsy may have detrimental effects on the developmental potential of the embryo. It has been reported that two of every five embryos that undergo cleavage stage biopsy, lose their ability to continue their normal development and implant. However, no such investigation has been carried out to study the effects of blastocyst biopsy on the viability and developmental potential (Scott et al., 2013)

6.3.2 Cleavage stage biopsy versus blastocyst stage biopsy (specific aim 1b)

This study set out to compare the time taken to reach full blastocyst, hatching rate and pregnancy outcome following cleavage stage biopsy or blastocyst stage biopsy. Results showed that significantly more embryos in the blastocyst stage biopsy group reached full blastocyst and hatching blastocyst compared to cleavage stage biopsy (p = <0.05). This observation can be supported by other studies that showed that performing blastocyst biopsy can be safer than cleavage stage biopsy. One potential explanation for this hypothesis is that the procedure involved in the removal of a smaller proportion of the embryo's total cellular content is less detrimental (Hardy et al., 1990). For instance, a single blastomere taken from 7 or 8 cells of embryo represents nearly 13% of the total content, however blastocyst biopsy can be performed by taking approximately 5 cells from the trophectoderm (200-300 cells at this stage) which represents only 2-3% of total cell content. Another contributing factor is that for blastocyst biopsy, extra embryonic (trophectoderm) cells are taken which is contributing to placental tissues following implantation. While cleavage stage biopsy requires taking only one blastomere, the lineage dictating the specific developmental fate cannot be predicted based on morphology, regardless of molecular genetic evidence that commitment occurs at the cleavage stage (hansis et al., 2003). As embryos at blastocyst stage have gone through genomic activation, they have better tolerance to manipulation compared to cleavage stage (Braude et al., 1988). However, although blastocyst and hatching rate was higher in blastocyst biopsy embryos then cleavage stage biopsy, no significant difference in implantation rate and pregnancy rate was found between these two groups in our study. This finding is in contrast to that of Scott and colleagues in 2013, who found that patients that went through blastocyst stage biopsy achieved significantly higher pregnancy rate (Scott et al., 2007). However, in our study, significantly more embryos biopsied on day 5 were frozen compared to those biopsied on

day 3. Those embryos that were frozen were not transferred, and therefore no further information is available regarding pregnancy outcome.

6.4   . Conclusions

In conclusion, although these results represent some interesting new insights, this study has some limitations which should be appreciated and therefore further study is required in order to draw more accurate conclusions. The first issue is that, interpretation of the data is being based on the assumption that the two groups are comparable. All biopsied embryos used in this study had less than 50% fragmentation, however the sample size between the two groups were not equal; 612 embryos from (137 patients) were used for day 3 biopsies and 220 embryos from 35 patients were included in the day 5 biopsy groups. Furthermore, there are a number of differences in treatment procedures between the two groups of patients assessed. Differences in cumulative FSH dose used, hormone treatment used and ratio of IVF/ICSI were apparent among the two groups. It is possible that these differences between the two groups might impact on the results and conclusions. Furthermore, it was not possible to include a non-biopsied control group in the study, and most embryos were derived from patients with fertility problems including recurrent miscarriage and IVF failures. As a result, most embryos were not of high developmental quality, which may have had a negative impact on our results.

In summary, cleavage stage biopsy results in delayed development to the 9 cell stage, however after this time, the duration of each developmental stage assessed is reduced, leading to significantly earlier development to blastocyst stage and hatching in comparison to embryos biopsied at the blastocyst stage.

7   General Discussion

This project was largely successful in the fulfilment of its specific aims in that technologies currently being used in IVF clinics were investigated and, in many cases, adapted to the porcine system; specifically:

1. An atlas of porcine sperm morphology was developed and is now in general use with our collaborating company JSR's laboratories. Our results indicated that the freezing process negatively affect boar sperm morphological features. It was suggested that there is a significant correlation between sperm morphological features and capacitation rate.

2. For the IVF procedure, the optimum concentration of semen has been determined.

3. Potential novel assays for boar fertility (sperm aneuploidy, nuclear organization and chromatin packaging) were investigated, the most promising being the CMA3 stain which may ultimately prove to be a powerful predictor of fertility in boars.

4. Finally, given the need in porcine IVP to biopsy the embryo before genotyping, morphokinetic time-lapse data from human IVF clinics suggested that embryo biopsy does not appear to have an adverse effect on the subsequent development of the embryo.

As mentioned from the outset, this project was part of a wider effort to develop and improve porcine IVF at the University of Kent and the data presented here in represented a piece in the puzzle within this context. Semen quality has an important impact on fertilisation potential, and therefore this thesis was focused primarily on the impact of boar semen quality on pig breeding. In this research, two groups of boar sperm were assessed; including those defined as high quality and poor quality semen based on their motility and morphological

features as determined by us at Laboratory. Sperm quality parameters such as concentration, morphology, capacitation rate, chromatin packaging and aneuploidy rate were analysed and the association of all parameters with sperm morphological features have been examined. It has been suggested that sperm morphological features impact embryo quality and fertilisation potential in human, therefore we focused on association with sperm morphological features and other semen quality parameters in this thesis. This research may help to improve fertilisation rate in pig IVF laboratory. The impact of the freezing process was assessed on boar sperm morphological features and results identified that the freezing process has a significantly negative impact on boar sperm morphology. We suggested that the underlying cause of this finding could be that the use of during the freezing process may damage the plasma membrane or alternatively that the freezing process itself may result in compromised sperm morphology. In light of this, and given the association between poor sperm morphology and reduced IVF success rates in humans (Ikawa et al., 2010) it is possible that cryopreserved boar sperm indicate lower fertilisation potential. As such, we developed our porcine IVF protocol using fresh boar semen rather than frozen semen.

In addition to this, the work presented here investigated the impact of boar sperm concentration on IVF success rates and found no significant difference using 10^6 sperm/ml compared to 10^4 sperm/ml. However, in this study, 245 oocytes were fertilised in total and therefore to validate these results, further studies are required. Future work should concentrate on investigations in to a broader range of sperm concentrations from different boars in order to make sure whether the optimum sperm concentration for fertilisation is also boar specific.

It was found that the optimum sperm incubation time and IVF media supplementation appears to be specific to the boar semen sample used and our results suggested that there

is no significant difference between fertilisation rate in the capacitation media with caffeine and adenosine supplements. It was indicated that no significant difference in the percentage of capacitated spermatozoa in the capacitation media with caffeine and adenosine supplement, therefore our results were in accordance with our IVF fertilisation rate. Although, the IVF experiment in this study was performed only with high quality boar sperm and we do not have any IVF data regarding poor quality semen quality in terms of fertilisation rate.

This thesis investigated the correlation between capacitation rate and boar sperm morphological features, it has been found that in poor quality boar semen group, the capacitation rate significantly decrease compare to high quality boar semen group. Therefore, it can be argued that the boar sperm morphological features can be considered as a capacitation rate indicator. The capacitation method can be applied in industry, as it is an inexpensive and quick technique to determine fertilisation potential. The role of sperm aneuploidy, chromatin packaging and nuclear organisation in the boar sperm head represent promising avenues for future study and, as only small numbers of boars were investigated it is feasible that associations with fertility problems may be established in other animals in future studies. By far the most promising avenue as a result of this thesis is the application of the CMA3 stain and we are in discussions with JSR about implementing this as a routine test.

In the future, it is highly plausible that porcine IVP will have significant economic, social and environmental benefits to the pig breeding industry, particularly in establishing breeding stocks to overseas farms. The work presented in this thesis provides the identification of, and comprehensive analysis of a number of factors that act to optimize sperm selection procedures and in vitro fertilization success. Furthermore, the ability to biopsy and determine

the sex of preimplantation embryos has been developed by other members within our laboratory and this will provide additional benefits to the pig breeding industry going forward. As such, the final chapter of this thesis analyzed the impact of embryo biopsy on human embryo development as an indicator of how such procedures may be relevant in the porcine model. This data provided a strong indication that our own biopsy strategies (which used the same standard operating procedures) could continue with confidence.

In general, this thesis was successful in studying the impact of boar semen quality on pig breeding and we could assess boar sperm efficiently in order to select high quality sperm for IVF. The work presented here indicated that sperm morphological features may be considered a strong indicator of capacitation and fertilisation potential and such evaluations improved our porcine IVF protocols and success rates considerably. In future, it would be beneficial to introduce two further methods for sperm selection (CMA3 staining and the sperm capacitation technique described in this thesis), both of which have clear industrial application. Future work should focus on performing fertilisation test according to both groups of morphologically high quality and poor quality semen, in order to ascertain about association between morphology and fertilisation rate.

8   Appendix

Chromosome1

n=385

p =   0.002

Percentage signal

Periphery   <<   Shell   >>   Interior

Chromosome 2

n= 380

p =<0.0001

Percentage signal

Periphery   <<   Shell   >>   Interior

Chromosome 3

n = 300
P=<0.0001

Periphery   <<   Shell   >>   Interior

Chromosome 6

n=392

p =   <0.0001

Periphery   <<   Shell   >>   Interior

Chromosome 7

n = 320
p = 0.0007

Percentage signal

35
30
25
20
15
10
5
0

1   2   3   4   5

Periphery   <<   Shell   >>   Interior

Chromosome 10

n=300
p = <0.0001

Percentage signal

45
40
35
30
25
20
15
10
5
0

1   2   3   4   5

Periphery   <<   Shell   >>   Interior

Figure 8.1. Autosomal chromosome positioning (chromosomes 1-18) in the sperm heads of three fertile boars following 2D analysis of FISH signals using DAPI density compensation models. Error bars represent the standard error of the mean, n is the number of sperm nuclei analysed and p is the statistical significance of non-random distribution assessed using a Chi-squared test.

chromosome 1

n=100

p =   0.325

Percentage signal

40
35
30
25
20
15
10
5
0

1   2   3   4   5

Periphery   <<   Shell   >>   Interior

Chromosome 2

n=100

P =0.064

Percentage signal

35
30
25
20
15
10
5
0

1   2   3   4   5

Periphery   <<   Shell   >>   Interior

Chromosome 3

n = 200
P=<0.0001

Periphery << Shell >> Interior

Chromosome 10

n= 200
p = <0.0001

Periphery << Shell >> Interior

Chromosome 12

n= 100

p =   0.027

Percentage signal

1   2   3   4   5

Periphery   <<   Shell   >>   Interior

Chromosome 14

n= 100

p =   <0.0001

Percentage signal

1   2   3   4   5

Periphery   <<   Shell   >>   Interior

Figure 8.2. The chromosome positioning for (chromosomes 1, 2, 3, 10, 12, 14, 16 and 18) in one sub-fertile boar. Error bars represents the standard error of the mean, n is the number of sperm nuclei analysed, p is the statistical significance for non-random positioning assessed by Chi-squared analysis, where p<0.05 indicates non-random positioning.

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