Influence of oocyte retrieval methods and maturation media on *in vitro* development of porcine embryos

by

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Declaration

I, Kayla Morgan Honneysett hereby declare that this dissertation titled: Influence of oocyte retrieval methods and maturation media on *in vitro* development of porcine embryos, submitted for the MSc(Agric) Animal Science: Reproduction Physiology degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

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Abstract

Oocyte recovery, *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) are reproductive technologies used to advance an oocyte to an embryo stage. The objectives of the study were to (1), compare oocyte retrieval methods (aspiration and slicing) from pig ovaries on oocyte quality and quantity; (2), compare IVM media (NCSU 37, TCM 199 and mpFF= porcine follicular fluid + FSH + LH) on oocytes polar body extrusion; (3), compare the effect of electrical stimulation (ES) and frozen-thawed semen on cleavage rates and (4) compare the effect of culture media, cNCSU 37 and cpFF, on zygote cleavage rates. Follicular fluid collected from the retrieval methods were assessed for the presence of oocytes using a microscope and categorized as Grades A, B or C. The IVM media were placed in a four-well multidish, thereafter Grades A and B oocytes were randomly allocated per treatment groups, matured for 44 hours and checked for polar body extrusion. Matured ootids were randomly allocated to either ES treatment or IVF treatment with frozen-thawed semen. The ES treatment used a medium consisting mainly of mannitol while BO-IVF was used for the frozen-thawed treatment. ES and IVF took place in conjuncture with *in vitro* culture (IVC), where both results were obtained following placement in IVC media. Zygotes were then checked and recorded for cleavage, single cell or lysed. The treatment means were compared using the Fisher's protected t-test least significant difference. Results showed significant differences with Grades A and B accounting for 50.8% of total oocytes (193.8) for aspiration and 58.7% of 488.6 for slicing. Oocytes' polar body extrusion was recorded as 25.3%, 84.2% and 73.8% for NCSU 37, TCM 199 and mpFF respectively. The percentage of cleaved zygotes for IVF with frozen-thawed semen was 48.1% and 56.8% for ES. The percentage of cleaved zygotes for cNCSU 37 was 66.6% and was 45.7% for cpFF. The slicing method proved to be better than aspiration for the retrieval of Grades A and B oocytes and the total number of oocytes retrieved. TCM 199 and mpFF media had a higher percentage of oocytes with polar body extrusion than NCSU 37. Cleavage rate of zygotes was higher when using ES and cNCSU 37.

Contents

List of Abbreviations

ANOVA	Analysis of variance
ARC	Agricultural Research Council
ART	Assisted reproductive technology
BSA	Bovine serum albumin
BO	Bracket and Oliphant
CA	Corpus albicans
CASA	Computer aided sperm analyser
cAMP	Cyclic adenosine monophosphate
CL	Corpus luteum
cNCSU 37	Modified North Carolina State University (culture)
COC	Cumulus oocyte complex
cpFF	Modified porcine follicular fluid (culture)
DAFF	Department of Agriculture
DDW	Deuterium depleted water
DOHaD	Developmental Origins of Health and Diseases
EGF	Epidermal growth factor
ES	Electrical stimulation/ activation
FBS	Foetal bovine serum
FSH	Follicle stimulating hormone
g	Grams
GCRB	Germplasm, Conservation, Reproduction and Biotechnologies laboratories
GF	Graafian follicle
h^2	Heritability
HbT	HEPES-buffered Tyrode's medium
ICAR	International Committee for Animal Recording
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IVC	*In vitro* culture
IVEP	*In vitro* embryo production
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
kg	Kilogram
LH	Luteinizing hormone
mDPBS	Modified Dulbecco's Phosphate Buffered Saline
ml	Millilitres
mm	Millimetres
MOET	Multiple ovulation embryo production
mpFF	Modified porcine follicular fluid (maturation)
N	Number

NCSU	North Carolina State University
PBS	Phosphate buffered saline
pFF	Porcine follicular fluid
PVA	Polyvinyl alcohol
R&D	Research and Development (R&D) Projects
SCA®	Sperm Class Analyser®
SOFM	Synthetic oviduct fluid medium
TALP	Tyrode's albumin lactate pyruvate
TBM	Tris-buffered medium
TCM	Tissue culture medium
TGEV	Transmissible gastroenteritis
µl	Microlitres

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Introduction

The use of assisted reproductive technologies (ARTs) in the porcine industry will allow for an increase in reproductive efficiency; as well as an increase in the productivity of the industry for the supply of pork products (Romar *et al.*, 2016, 2019). While pork products are highly consumed in other countries, in South Africa it is not a popular source of protein (Davids *et al.*, 2013). The reason for the lack of popularity of pork could be contributed to specific religious preferences of consumers within South Africa, the high cost of meat due to high production costs and importation of pork from other countries (Robinson, 2018). Pork products have been shown to be a valuable food source, but due to its lack of popularity in South Africa it is not viewed as such. Therefore once it is recognised for its value, the demand for pork products will begin to increase (Tait-Burkard *et al.*, 2018; Romar *et al.*, 2019). This shows that there is an opportunity to improve the efficiency of pig production in South Africa to decrease the importation of pork and thereby become more self-sufficient. This will allow for the average cost of pork to decrease and become more affordable for the poorer communities in order to become a more popular source of protein.

The application of ARTs in *in vitro* embryo production (IVEP) can be defined as successful when immature oocytes are recovered, matured and fertilized to reach the blastocyst stage post culturing (Nedambale *et al.*, 2004; Lekola, 2015). The use of IVEP technologies are used in circumstances where multiple ovulation and embryo transfers (MOET) cannot be utilized; such as when using prepubertal females, gestating or slaughtered animals for reproduction purposes (Armstrong *et al.*, 1992, 1994; Hasler, 1994; Baldassarre *et al.*, 1996).

The efficiency of using ARTs for porcine germplasm has been shown to be lower than in other species, especially in comparison to cattle (Kikuchi *et al.*, 2002; Schoevers *et al.*, 2003; Sutton-McDowall *et al.*, 2006; Garcia-Garcia *et al.*, 2007; Gutnisky *et al.*, 2007; Zhu *et al.*, 2007; Alvarez *et al.*, 2009). This can be seen through the success rate following *in vitro* fertilization (IVF), with that in porcine being approximately 45% while *in vitro* production in cattle has a 70% success rate (Rizos *et al.*, 2008; Romar *et al.*, 2016, 2019). Gil *et al.* (2010) found a 30-50% efficiency in porcine IVF which is lower compared to cattle, because polyspermic embryos develop to the blastocyst stage at the same rate as healthy embryos. Therefore reproductive technologies that are currently used for porcine germplasm have drawbacks due to the high rate of polyspermy which leads to poor development rates and low quality blastocysts (Nagai *et al.*, 2006; Gil *et al.*, 2010; Pyoos *et al.*, 2018). It has been shown that embryo transfers from embryos that have been cultured *in vitro* can result in low pregnancy rates and high embryo mortality rates (Funahashi *et al.*, 1997a; Long *et al.*, 1999).

The *in vitro* production of porcine embryos is less effective than *in vivo* embryos (Papaioannou & Ebert, 1988; Machaty *et al.*, 1998; Kikuchi *et al.*, 1999; Yoon *et al.*, 2000; Tatemoto *et al.*, 2004). Advancing the use of porcine reproductive technologies will allow for better results and for further use of other technologies. Embryo transfers, instead of live animals, cater for adaptation as embryos brought from other countries can be implanted into females that are already adapted to the South African environment. This will give maternal passive immunity to the embryos received from other countries, or other regions within South Africa, following parturition which will improve the efficiency of the imported genotype (Wagstrom *et al.*, 2000).

By improving the technologies used for *in vitro* procedures the efficiency of production of porcine embryos in South Africa can improve. This would be particularly important for the South African pork industry as there is currently little information on how to use these biotechnologies within the farming community in order to close the pork consumption gap with other countries.

1.2 Aim

The aim of this study was to investigate how to improve the maturation, fertilization and culturing media and techniques for porcine embryos produced *in vitro*.

1.2.1 Hypotheses

The hypotheses were then aligned with the four main aims of the study. (H_1) The collection techniques, slicing and aspiration, will have the same effect on the quality of porcine oocytes when recovered. (H_2) Oocyte maturation rate will be the same when using the three different *in vitro* maturation media. (H_3) Electrical stimulation and frozen-thawed semen will have the same fertilization ability following IVF. (H_4) The two culture media will have the same effect on embryo cleavage rates.

1.3 Objectives

The specific objectives of the study were:

1) To determine the effect of retrieval techniques (slicing and aspiration) on the quality of porcine oocytes

2) To compare three different *in vitro* maturation media (NCSU 37, TCM 199 and follicular fluid + FSH & LH) on porcine oocyte maturation rates

3) To compare electrical stimulation on oocytes and frozen-thawed semen on fertilization rates following IVF

4) To compare two *in vitro* culture media (cNCSU 37 and cpFF) on embryo cleavage rates

Chapter 2: Literature Review

2.1 Introduction

Worldwide, pork is the top produced meat and is highly consumed, but in South Africa pork is consumed ten times less than poultry. High feed costs may be partially responsible, which account for 70-80% of total farm costs, as well as only 200 000 tons of pork is being consumed per year (Davids *et al.*, 2013). If the consumption of pork products can be increased by tenfold the potential to improve upon the pork industry is great (Robinson, 2018). South Africa produces 182 000 tons with the rest of the required tonnage imported from Germany, France, Spain, Canada, Brazil and USA. While countries that South Africa exports to include Namibia, Mauritius and Mozambique (Robinson, 2018). Therefore there is an opportunity to improve the efficiency of the pig production industry in South Africa. The production of pork is fairly spread out over the country, with three provinces producing the highest number of sows (Table 2.1). This will allow for a decrease in the transportation of pork, if proper equipped abattoirs and meat packing facilities are close by. These factors could allow the country to become more efficient in the production of pork. The average consumer cost of pork can then decrease and will become more affordable for the poorer communities in order to become a more popular source of protein.

Table 2.1 The number of sows per province in South Africa (SAPPO, 2019)

Province	**Number of sows**
Northwest	17 300
KwaZulu-Natal	16 600
Western Cape	16 000
Limpopo	13 700
Gauteng	13 300
Mpumalanga	10 500
Free State	9 000
Eastern Cape	4 600
Northern Cape	2 000
Total	103 000

Improving reproductive technologies in the porcine industry can help achieve a handful of sustainable development goals (SDGs) set for 2030 (UNDP, 2019). These include, but are not limited to: Goal 1: no poverty- improving the production system will allow for an increase in jobs and will improve the profit value throughout the entire system (UNDP, 2019). Goal 2: no hunger- as the industry improves and grows more food will be available for consumption (UNDP, 2019). Goal 5: gender equality- this is not limited to any specific gender having an advantage for the work needed and so

opens up job opportunities for both men and women (UNDP, 2019). Goal 12: responsible consumption and production- using this technology does not cause any damage or strain on the environment (UNDP, 2019). Goal 13: climate action- greenhouse gases are one of the worlds' biggest threats. Assisted reproductive technologies (ARTs) aids in the improvement of reproductive efficiency and can decrease generation interval. This technology could improve the number of piglets carried per parturition and so decrease the resources required to produce the same number of piglets as those naturally produced (UNDP, 2019). The study will contain the ARTs: *in vitro* maturation, *in vitro* fertilization and *in vitro* culture.

There are three main sectors in the South African pork industry, which are the large scale, middle and small commercial producers. The large scale commercial sector is intensive with units consisting of 600- 5000 sows. It may be more beneficial to have a large scale commercial system with a high output rate; as input costs are expensive, with 75% of the costs go towards buying feed (Robinson, 2018). Improving the use of reproductive technologies in the South African commercial pork industry will help increase production and therefore profit (Pyoos *et al.*, 2018). This is due to the commercial farms having better access to genetic diversity when correctly using the available technology (Pyoos *et al.*, 2018). Current reproductive technologies being used have drawbacks as they have a high rate of polyspermy as well as low development rates and poor blastocyst quality (Gil *et al.*, 2010). Current IVF efficiency is only 30-50% as porcine embryos that are polyspermic will develop the same as healthy embryos to the blastocyst stage (Gil *et al.*, 2010). Having poor efficiency of developing healthy embryos in swine is an issue with the application of gene transfer and other biotechnologies (Bearden *et al.*, 2004). *In vitro* embryo production (IVEP) can be determined to be successful when immature oocytes can be recovered, then matured and fertilized in order to reach the blastocyst stage post culturing (Nedambale *et al.*, 2004; Lekola, 2015). Although differences can be found between *in vivo* and *in vitro* produced embryos, this can also be determined by factors such as oocyte quality, follicular environment, fertilization methods and culture environment (Ferre *et al.*, 2002; Nedambale *et al.*, 2004; Camargo *et al.*, 2006; Lekola, 2015). The use of IVEP technologies can be used for successful reproduction in circumstances where multiple ovulation and embryo transfers (MOET) cannot be utilized; such as prepubertal females, gestating or slaughtered animals (Armstrong *et al.*, 1992, 1994; Hasler, 1994; Baldassarre *et al.*, 1996). Although, it has been shown that embryo transfers from embryos that have been cultured *in vitro* can result in low pregnancy rates and high embryo mortality rates (Funahashi *et al.*, 1997a; Long *et al.*, 1999).

Furthermore, reproductive technologies can be used to conserve genetic resources (Pyoos *et al.*, 2018). *In vitro* production of porcine embryos has shown to be less effective than *in vivo* embryos (Papaioannou & Ebert, 1988; Machaty *et al.*, 1998; Kikuchi *et al.*, 1999; Yoon *et al.*, 2000; Tatemoto *et al.*, 2004). Advancing the use of reproductive technologies will allow for better results and for further use of other technologies. Using cryopreservation to store germplasm, in the form of a biological bank,

can be used to preserve cells and tissues for future use and possible species rescue strategies (Silva *et al.*, 2015). The storing of germplasm can then be utilized to prevent the extinction of certain species (Silva *et al.*, 2015). Cryopreservation can further be used to export desired genotypes to different parts of the world (Berthelot *et al.*, 2003). Sending germplasm instead of live animals can be beneficial in terms of new genetics availability, decreasing disease transmission and is cost/time efficient (Bearden *et al.*, 2004). Embryo transfers, instead of live animals, cater for adaptation as embryos brought from other countries can be implanted into females that are already adapted; this will give maternal passive immunity to the embryos after parturition to improve the efficiency of the imported genotype (Wagstrom *et al.*, 2000).

2.2.1 Maternal passive immunity

Porcine placenta can be defined as diffuse epitheliochorial, this type of placental connection does not allow for the transfer of immunoglobulins from the pregnant female to the foetus (Kortbeek-Jacobs *et al.*, 1984; Wagstrom *et al.*, 2000). Therefore the piglet is born agammaglobulinemic and would need to receive immunoglobulins from the milk provided by the sow (Bourne, 1977; Wagstrom *et al.*, 2000). Colostrum is important for all neonatal species, with the main immunoglobulin component in pigs being immunoglobulin G (IgG). The piglets receiving colostrum after birth are given a source of circulating antibodies and thereafter when receiving milk, with the main immunoglobulin component being immunoglobulin A (IgA), they are provided with local antibody protection and local passive immunity (Bourne, 1977; Wagstrom *et al.*, 2000). Passive immunity can be described when piglets nurse at regular intervals from immune sows in order to gain the protection of the epithelial cells of the small intestine; this will allow the suckling piglets to then be protected against the prevalent environmental infections or have a less severe reaction to a possible infection (Bohl *et al.*, 1972).

It has been shown that when a pregnant female was exposed to certain orally administered antigens post farrowing, she had lymphocytes that would preferentially produce sensitized IgA (Kortbeek-Jacobs *et al.*, 1984). Bourne (1977) showed when exposing a pregnant female to live *Escherichia coli* the milk then produced by the female had high levels of anti-*E. coli* IgA present. Bohl *et al.* (1972) found that when the gastrointestinal tract of a pregnant sow was infected with transmissible gastroenteritis (TGEV) the milk that was produced had IgA present that worked against the disease. The timing of colostrum ingestion is important in making sure the piglet receives the adequate antibodies in order to have an environmentally adapted active immune system. The piglets' intestinal epithelium is highly absorptive of macromolecules such as IgG for the first 24 to 36 hours, thereafter the absorption ability will decrease until gut closure occurs (Wagstrom *et al.*, 2000).

Recent studies have been focussing on the epigenetic effects on pregnant females and the foetus. A new research field called Developmental Origins of Health and Diseases (DOHaD) has been observing the effect of environmental and maternal factors on the health of the offspring (Chavatte-

Palmer *et al.*, 2018; Carvalho *et al.*, 2020). It has been shown that environmental factors that animals become exposed to may have an influence on the animal itself as well as their subsequent generation/s, this can be explained by epigenetic transgenerational inheritance studies (Skinner, 2011; Thompson *et al.*, 2020). Not only is a pregnant female exposed to environmental stressors, the foetus she is carrying as well as the foetus' own germline is also exposed (Thompson *et al.*, 2020). This will allow for the next three generations (F1-F3) to be more adapted to the environment that they are born into, in the event of a F1 female reproducing in the specific environment (Skinner, 2011).

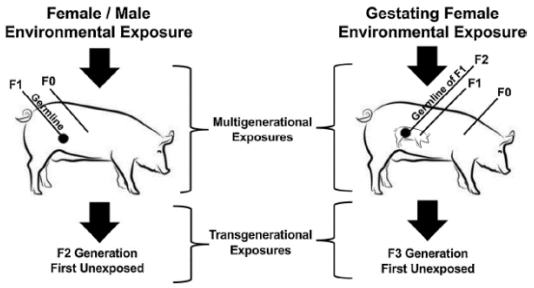

Figure 2.2.1.1 Environmental exposure on pigs and their subsequent generations (Skinner, 2008; Nilsson *et al.*, 2018; Thompson *et al.*, 2020)

For the relationship between environmental effects, such as nutrition and toxicants, on the pregnant sow and the effect on the epigenetic machinery of the foetus, which further influences the phenotype, there is little information available (Chavatte-Palmer *et al.*, 2018; Thompson *et al.*, 2020). Though some studies have been performed to determine how the nutritional environment prior to birth influences the expression of genes and the offspring's performance post birth (Chavatte-Palmer *et al.*, 2018).

2.2.2 Transportation and treatment

It is vital to find the correct transportation methods of ovarian tissue to ensure adequate follicle survival and oocyte viability. The precise specifications for the optimal temperature and the maximum duration of transport is unknown. Previous studies done on transportation have shown that ovaries can be placed in a broad temperature range of 4-39°C for 1-24 hours, but it has been theorized that as the temperature increases the time of transport must decrease (Barberino *et al.*, 2019; Gadea *et al.*, 2020). Weissman *et al.* (1999) showed that the survival and development of follicles were increased when transporting in warm medium rather than cold, specifically over a short time period. The body temperature of a pig is 38-40°C, it may then be deduced that transportation of ovaries must happen at a temperature equal to, or close to natural body temperature.

Different types of follicles present on the ovary may react differently to transport environment. Primordial follicles are more susceptible to degeneration than primary follicles, this is due to the fact that primordial follicles are dormant, and may be more sensitive to changes, while primary follicles are

activated and have already begun growth (Raffel *et al.*, 2020). Although as transportation time increases the number of follicles that degenerate will increase; with a significant increase in degenerated follicles occurring after 4 hours of transportation at body temperature (Raffel *et al.*, 2020).

Ovaries obtained from prepubescent gilts are more commonly used as gilts make up the majority of slaughter pigs in developed countries (Marques *et al.*, 2015; Gadea *et al.*, 2020). Ovaries must be collected and transported to the intended work site within an hour of slaughter. Ovaries are to be transported in a thermos flask in 0.85-0.9% NaCl saline solution, with the option to include an antibiotic, and kept at a constant temperature of 39°C to decrease the risk of degeneration and contamination (Hamano & Kuwayama, 1993; Yoon *et al.*, 2000; Kumar *et al.*, 2016; Okamoto *et al.*, 2016; Pyoos *et al.*, 2018; Gadea *et al.*, 2020).

2.2.3 Oocyte recovery

Oocyte recovery methods have been largely developed because oocytes used in studies are taken from ovaries that have been collected from prepubescent gilts from the slaughter house (Marques *et al.*, 2015; Gadea *et al.*, 2020). Ovaries from slaughter houses offer a copious and cheap amount of oocytes for use in *in vitro* studies and production (Kumar *et al.*, 2016). The main recovery methods used on slaughter tissue is aspiration and slicing. Due to the tissue being taken from prepubescent females this makes certain recovery methods more difficult; with the process of aspiration entailing the removal of follicular fluid from within the follicles. If the follicles are small, as found in young females, then the process becomes more time consuming and the slicing method then becomes the oocyte removal method of choice (Marques *et al.*, 2015).

Prior to retrieval methods, the ovaries are prepared by removing the tissue debris and washing in phosphate buffer saline (PBS). Ovaries have a multi-lobulated shape and can be evaluated by recording the weight, length and breadth as well as the number of Graafian follicles (GF), corpora lutea (CL) and corpora albicans (CA) present (Kumar *et al.*, 2016). Ovaries are held in saline solution at room temperature until needed for oocyte removal (Long *et al.*, 1999). The aspiration process entails the use of an 18 Gauge needle on a 5ml or 10ml syringe being inserted into all visible follicles of 2-6mm to remove the follicular fluid and the oocytes within (Hamano & Kuwayama, 1993; Yoon *et al.*, 2000; Marques *et al.*, 2015; Okamoto *et al.*, 2016; Pyoos *et al.*, 2018). During the slicing process the ovaries are placed in a petri dish containing a medium, HEPES-buffered Tyrode's medium (HbT) or a TCM-199 medium with 5% foetal bovine serum (Hamano & Kuwayama, 1993; Marques *et al.*, 2015). A single ovary is held in place with hemostatic forceps and follicles of 3-6mm are sliced with a scalpel blade (Marques *et al.*, 2015; Okamoto *et al.*, 2016; Gadea *et al.*, 2020). Hamano & Kuwayama (1993) used a similar cutting method but used ten razors set apart at 2mm intervals to slice the ovary instead of a single scalpel. It was shown by Hamano & Kuwayama (1993) that the slicing technique was

superior to the aspiration method but there is no difference in the ability to advance to the blastocyst stage.

After both recovery techniques the fluid that had been collected is placed in 50ml tubes and placed in a water bath at 35°C for 15 minutes in order to allow the cumulus oocyte complexes (COCs) to settle and a supernatant to form (Marques *et al.*, 2015). This process is then repeated twice and the COCs can then be examined under the microscope (Marques *et al.*, 2015). Grading of oocytes can then be done under the microscope by examining the layers of cumulus cells surrounding the cell. A grade I oocyte will have 6 layers of cumulus cells, a grade II oocyte will have 3-5 layers, a grade III oocyte will have 1-2 layers and be partially denuded and a grade IV oocyte is completely denuded and lacks any layers of cumulus cells (Hamano & Kuwayama, 1993; Khandoker *et al.*, 2001; Lekola, 2015; Kumar *et al.*, 2016).

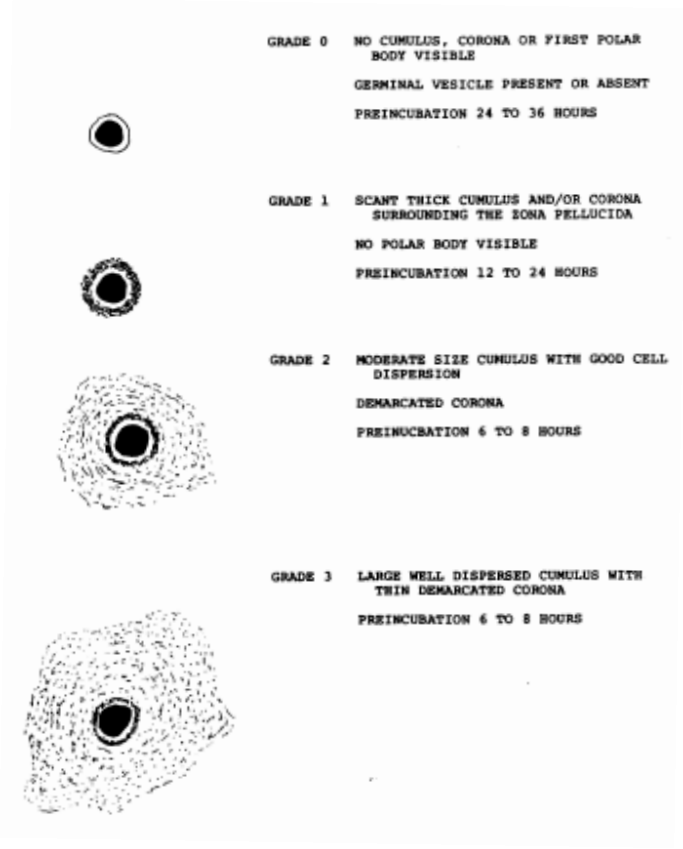

Figure 2.2.3.1 The oocyte grading system (Marrs *et al.*, 1984)

The cytoplasm of the cell, the ooplasm, should have a dark and granulated appearance (Yoon *et al.*, 2000; Gadea *et al.*, 2020). The presence of cumulus cells around the oocyte have an important role through influencing a paracrine communication on the development on the oocyte (Assidi *et al.*, 2011; Demiray *et al.*, 2017). Cumulus cells have also been found to protect the oocytes from oxidative stress due to *in vitro* conditions having higher levels of oxygen than the *in vivo* environment (Tatemoto *et al.*, 2000, 2004). Kumar *et al.* (2016) found that season had an impact on the presence of specific

grades of oocytes, with grade I oocytes making up 68% of collected oocytes during the winter season and grade IV oocytes to be in high abundance during the warmer seasons when the female is under heat stress.

2.2.4 *In vitro* maturation (IVM)

In vitro maturation (IVM) is a reproductive technology used to advance an oocyte to a maturation stage, thereafter it may be used for *in vitro* fertilization (IVF). A "true ovum" never exists unless in a transient state or when a second maturation division occurs and completes before fertilization (Bearden *et al.*, 2004). An oocyte is considered mature when it reaches metaphase II and the first polar body is formed (Lekola, 2015; Pyoos *et al.*, 2018). The polar body is formed following the division of the nucleus of the oocyte, the germinal vesicle (Lekola, 2015). In mammals a surge of progesterone or luteinising hormone (LH) levels will stimulate the progression from prophase I to metaphase II (Jahn & Sudhof, 1999; Lekola, 2015).

The size of the follicle from which an oocyte is taken and the presence of follicular fluid influences the ability of the oocyte to develop and mature (Vatzias & Hagen, 1999; Pyoos *et al.*, 2018). Oocytes removed from large follicles (>5mm) have been shown to have a better chance of maturing than a small follicle (<3mm) as smaller follicles are not fit for meiosis and unable to undergo complete cytoplasmic development (Marchal *et al.*, 2002b; Gil *et al.*, 2010). With IVM, oocyte maturation should include nuclear and cytoplasmic maturation but cytoplasmic maturation can be imperfect as the IVM conditions can stop the movement of mitochondria to the oocytes inner cytoplasm, this will then affect the maturation ability (Gil *et al.*, 2010). Before placement in maturation media the oocytes have to be washed to remove any debris that was collected along with them during oocyte removal. Oocytes can be washed three times using a bicarbonate-buffered or HEPES buffered TCM 199 that is further supplemented with 10% foetal bovine serum (FBS) or using a HEPES buffered Tyrode medium supplemented with 0.01% polyvinyl alcohol (PVA) (Baldassarre *et al.*, 1996; Tatemoto *et al.*, 2004; Karami Shabankareh *et al.*, 2011).

The use of maturation media are an important choice to make as it can impact the growth of the oocytes. The media used should contain chemicals that will have the highest positive effect on oocyte maturation and subsequent embryo development. The use of gonadotrophic hormones in maturation media could increase the rate of development of oocytes *in vitro* through simulating an *in vivo* environment. The more common maturation media used are NCSU 37 (Table 2.2.4.1), NCSU 23 (Table 2.2.4.2) and TCM199 (Table 2.2.4.3) (Gil *et al.*, 2010). Pyoos *et al.* (2018) found that NCSU 37 had a high rate, 81.9-85.9%, of maturation and polar body formation when compared to NCSU 23. These media was supplemented with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) respectively. While NCSU 23, which was found to not show significance from the

control, was supplemented with follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Pyoos *et al.*, 2018).

Table 2.2.4.1 The composition of NCSU 37 maturation media (50ml) (Pyoos *et al.*, 2018)

Constituent	**Concentration**
Stock solution A	36 ml
Stock solution B	9 ml
PFF	5 ml
Glucose	0.0045 g
Sorbitol	0.0984 g
B-ME stock solution	50 µl
L-Cysteine stock solution	500 µl
Antibiotic (Antimycotic 2%)	1 ml
dbcAMP stock solution	200 µl
PMSG (pregnant mare serum gonadotropin)	10 µl
hCG (human chronic gonadotropin)	10 µl

PFF= porcine follicular fluid

Table 2.2.4.2 The composition of NCSU 23 maturation media (50ml) (Pyoos *et al.*, 2018)

Constituent	**Concentration**
Stock solution A	36 ml
Stock solution B	9 ml
PFF	5 ml
Glucose	0.0045 g
Sorbitol	0.0984 g
B-ME stock solution	50 µl
L-Cysteine stock solution	500 µl
Antibiotic (Antimycotic 2%)	1 ml
FSH	100µl
LH	10µl

PFF= porcine follicular fluid, FSH= follicle stimulating hormone, LH= luteinizing hormone

Table 2.2.4.3 The composition of TCM 199 maturation media (Funahashi & Romar, 2004; Romar *et al.*, 2016)

Constituent	**Concentration (mM)**
NaCl	116.35
KCl	5.36

$MgSO_4$	0.81
NaH_2PO_4	1.01
Glucose	3.05
$NaHCO_3$	26.19
Caffeine	5.00
Ca-(lactate)$_2$	2.92
Na-pyruvate	0.91
$CaCl_2.2H_2O$	1.80
Sorbitol	12.00
Penicillin G/ streptomycin	0.17/0.07
BSA (bovine serum albumin)	4.00 mg/ml

However, it was found that media supplemented with concentrations of FSH and LH of 0.5-2.5µg/ml and 0.5-5 or 6µg/ml respectively showed higher maturation rates of oocytes and polar body extrusion (Dode & Graves, 2001; Lekola, 2015; De Macedo *et al.*, 2019). While Okamoto *et al.* (2016) used a concentration of 1:1000 of FSH in the maturation medium and Silvestre *et al.* (2007) found that when using a concentration of 0.05-0.5ng/ml there was no notable differences in embryo development. FSH released from the pituitary gland influences the growth of an antrum during follicular development and few antral follicles will progress into preovulatory follicles (Fortune, 1994; Bussalleu *et al.*, 2008; Okamoto *et al.*, 2016). FSH was shown to influence cumulus cells to produce cyclic adenosine monophosphate (cAMP) which thereafter influences oocyte maturation (Eppig, 1991; Lin *et al.*, 2011; Lekola, 2015). FSH was found to influence cumulus cell expansion and resumption of maturation, as well as prompting an increased effect of epidermal growth factor (EGF) on maturation (Singh *et al.*, 1993, 1997). Downs (1989) reported that the issue of meiotic arrest in oocytes could be resolved with the use of EGF as well as stimulating cumulus cell expansion. This growth factor's role in maturation could show a physiological link with the porcine ovary and specificity in preovulatory processes (Singh *et al.*, 1995, 1997). Pyoos *et al*. (2018) found that supplementing EGF with beta-mercaptoethanol and hypotaurine improved blastocyst rates.

LH was shown to enhance cleavage and therefore improve embryonic development and competency (Pyoos *et al.*, 2018). Although LH has been found to not directly influence maturation but instead to influence the release of paracrine factors by granulosa cells that play a role in oocyte maturation (Figure 2.2.4.1) (Nuttinck *et al.*, 2004; Lekola, 2015).

The use of porcine follicular fluid (pFF) during the IVM stages may decrease the cost of IVM media and allow for this reproductive technology to be used by those that may not be able to afford the chemicals needed for other more popular IVM media but will also resemble the oocytes natural *in vivo* conditions (Agung *et al.*, 2013). pFF is removed from follicles of 2-6mm using the aspiration method,

it was then centrifuged at 10000 xg for 15 minutes, or 2000 rpm for 10 minutes, at 4°C to form a supernatant that is removed and stored at -20°C to -30°C (Naito *et al.*, 1988; Tatemoto *et al.*, 2004; Karami Shabankareh *et al.*, 2011). Using pFF in an IVM medium has been shown to have a positive effect on nuclear maturation rate, fertilization and embryo cleavage following fertilization (Yoshida *et al.*, 1992; Tatemoto *et al.*, 2004). The resumption of meiosis and male pronucleus formation have been positively affected by the presence of pFF, especially when used in conjunction with FSH (Naito *et al.*, 1988; Rath *et al.*, 1995; Tatemoto *et al.*, 2004). It has been found that follicular fluid present in preovulatory follicles has a high concentration of estradiol (Funston *et al.*, 1996; Kojima *et al.*, 2003).

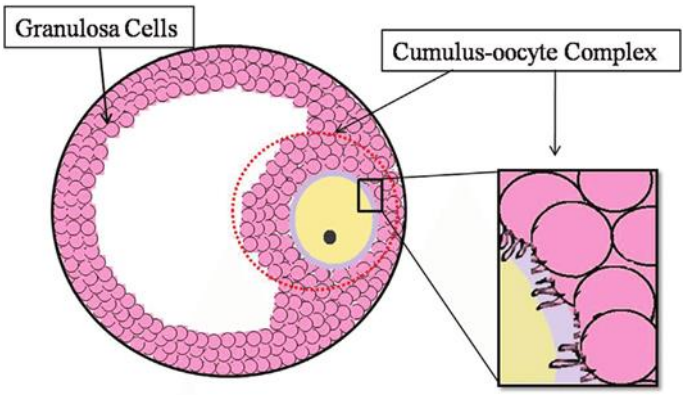

Figure 2.2.4.1 A schematic drawing of a cumulus oocyte complex (Demiray *et al.*, 2017)

Media used for maturation should be filtered with a 0.22µl sterilizing filter except media containing FSH and/ or LH (Karami Shabankareh *et al.*, 2011). 100µl of the maturation medium is prepared and covered with mineral oil, the oocytes are then placed in the medium at 39°C with 5% carbon dioxide gas for 48 hours (Tatemoto *et al.*, 2004; Okamoto *et al.*, 2016; Demiray *et al.*, 2017; Pyoos *et al.*, 2018). Alternatively, roughly around 50 oocytes can be placed in 500µl of maturation medium (Long *et al.*, 1999). When the maturation medium contains FSH, of approximately 100ng/ml, the oocytes are matured for 24 hours, then placed in fresh media of the same specifications and matured for a further 24 hours (Okamoto *et al.*, 2016). EGF can be used to initially mature the oocytes for 48 hours and supplemented with bovine serum albumin (BSA) (Pyoos *et al.*, 2018). Yamauchi *et al.* (1996) found that timing of maturation is important, their results showed higher rate of oocyte maturation occurred at 42 and 48 hours. Further, the oocytes that matured for 42 hours had a higher activation percentage after IVF but this percentage started decreasing when allowed to mature for 48 hours (Yamauchi *et al.*, 1996). When using porcine follicular fluid as a maturation media (MpFF) the timing of 44 to 48 hours is used (Agung *et al.*, 2013).

2.2.5 Semen quality analysis

Semen quality can be determined by multiple factors, those being: morphology, motility, concentration and viability (Tsakmakidis *et al.*, 2010). High rates of semen concentrations can cause issues in the IVF stage as it could lead to polyspermy. Therefore using diluted semen, at $3x10^7$

spermatozoa/ml for example, can increase the ability to fertilize successfully (Xu *et al.*, 1996; Gadea & Matas, 2000).

Sperm morphology being normal as well as having a progressive forward motility has been shown to have a mostly positive effect on boar fertility (Ivanova & Mollova, 1993; Xu *et al.*, 1996; Flowers, 1997; Gadea & Matas, 2000; Tsakmakidis *et al.*, 2010). Where motility, velocity and direction of movement being the biggest indicators of spermatozoa quality (Mortimer *et al.*, 2015; Mphaphathi, 2017).

The computer-aided sperm analyser (CASA) is used to determine the motility and velocity of spermatozoa within a semen sample (Mphaphathi, 2017). The CASA-SCA® system has become widely used for the analysis of semen quality. It has been shown that the quality determined by the CASA-SCA® system is highly correlated to the fertilization ability *in vivo* (Holt *et al.*, 1997; Mphaphathi, 2017).

2.2.6 *In vitro* fertilization (IVF) and electrical stimulation (ES)

Meiosis II *in vivo* is initiated during fertilization and won't be completed until interaction between the sperm and the oocyte (Bearden *et al.*, 2004). When fertilization occurs, the products from the second meiotic division are the zygote and the second polar body (Bearden *et al.*, 2004). Studies on *in vitro* fertilization (IVF) are done with the sole purpose to improve upon the poor success rates, compared to *in vivo*, within porcine embryo production (Romar *et al.*, 2016).

One of the issues within porcine IVF studies is the high incidence of polyspermy (Agung *et al.*, 2013; Romar *et al.*, 2016). High rates of polyspermy in porcine IVF systems may be due to a slow zona pellucida and cortical reaction which then allows for multiple sperm penetration (Sun *et al.*, 1992; Wang *et al.*, 1998a; Funahashi & Romar, 2004). Prior to semen use in IVF studies, washing of the semen takes place, by using a centrifuge, in order to separate the spermatozoa from the seminal plasma and extenders (Cheng *et al.*, 1986; Nagai *et al.*, 1988; Mattioli *et al.*, 1989; Abeydeera & Day, 1997; Funahashi *et al.*, 1997b; Romar *et al.*, 2016). There are two current centrifuge processes used for sperm preparation, the Percoll gradient centrifugations and the swim-up procedure (Clarke & Johnson, 1987; de Vries & Colenbrander, 1990; Horan *et al.*, 1991; Guthrie & Welch, 2006; Yeste *et al.*, 2009; Holt *et al.*, 2010; Romar *et al.*, 2016). The Percoll gradient process allows for selection of morphologically superior spermatozoa and can increase sperm penetration rates, cleavage and the rate of blastocyst development (Grant *et al.*, 1994; Jeong & Yang, 2001; Matas *et al.*, 2003, 2011; Caballero *et al.*, 2004; Romar *et al.*, 2016; Volpes *et al.*, 2016). The swim-up process allows for highly motile spermatozoa to swim into the fertilization medium, placed on top of the sperm pellet following centrifuging, this process reduces the occurrence of polyspermy (Clarke & Johnson, 1987; Park *et al.*, 2009; Gil *et al.*, 2010; Holt *et al.*, 2010; Romar *et al.*, 2016; Volpes *et al.*, 2016).

Making use of antioxidant supplements, colloids and/ or an iodixanol cushion when preparing cryopreserved spermatozoa for IVF can improve the quality and the capability to fertilize the ootids (Matas *et al.*, 2007; Zhang *et al.*, 2012; Martinez-Alborcia *et al.*, 2013; Romar *et al.*, 2016). Caffeine can be used when COC's are mixed with spermatozoa in order to decrease the incidence of polyspermy by 40%, as caffeine influences sperm capacitation and may induce spontaneous acrosome reaction (Funahashi *et al.*, 2000a; b; Funahashi & Romar, 2004; Romar *et al.*, 2016). The continual use of caffeine during culture could increase the rate of polyspermy but if a caffeine free medium is used then no sperm penetration will occur (Funahashi & Romar, 2004). The use of oviductal proteins and the preincubation of ootids with oviductal fluid could decrease the rate of polyspermy. This is due to their effect on the cortical reaction following movement into the perivitelline space (Kim *et al.*, 1996, 1997; Romar *et al.*, 2016).

Semen from fertile boars is used to ensure fertilization will occur. The use of frozen-thawed semen over fresh semen will result in data that is easy to reproduce as there is less of an individual boar effect on the results (Martinez *et al.*, 1993; Suzuki *et al.*, 2005; Gil *et al.*, 2010). Prior to use, the frozen-thawed spermatozoa must be preincubated at 37°C for 15 minutes (Nagai *et al.*, 1988; Agung *et al.*, 2013). Semen is placed in the IVF medium with 2ml/mL BSA and centrifuged at 2000-2400rpm for 2-3 minutes, due to boar spermatozoa having the capacity to withstand a high g force for a short amount of time (Carvajal *et al.*, 2004; Romar *et al.*, 2016; De Macedo *et al.*, 2019). The supernatant is then removed and the sperm pellet is diluted with the IVF media to make a concentration of $1x10^5$-$5x10^6$ sperm/ml (Baldassarre *et al.*, 1996; Wang *et al.*, 1998b; Agung *et al.*, 2013).

The common media used for IVF are Tris-buffered medium (TBM), tissue culture medium 199 (TCM 199) and Tyrodes albumin lactate pyruvate (TALP) (Abeydeera & Day, 1997; Wang *et al.*, 1998b; Isom *et al.*, 2012; Romar *et al.*, 2016). Porcine gamete media is commonly used but often in conjuncture with porcine oocyte medium (for IVM) and porcine zygote medium (for IVC) (Romar *et al.*, 2016). Funahashi & Romar (2004) used a modified TCM 199 media supplemented with Earles salts, sugars, antibiotics, simple organic acids and BSA.

Before IVF the ootids were washed twice in 10µl of the IVF media, five ootids per drop, and then placed in 40µl drops of the fertilisation medium where a further 10µl of semen solution will be added (Baldassarre *et al.*, 1996). Sperm penetration occurs two hours following ootid exposure to frozen-thawed sperm which triggers the resumption of meiosis (Marchal *et al.*, 2002a; Gil *et al.*, 2010). The oocyte-sperm mixture is placed in an incubator at 37-39°C with 5% CO_2, 7% O_2 and 88% N_2 for 24 hours before being later placed in an IVC medium (Baldassarre *et al.*, 1996; Demiray *et al.*, 2017). After the 24 hours of co-incubation the zygotes are removed from the IVF medium and separated from the spermatozoa and are then to be placed in an IVC medium (Yoshioka *et al.*, 2002; Agung *et al.*, 2013). Sperm penetration was determined to be successful when there was the presence of sperm heads

in the ooplasm or multiple pronuclei and a second polar body, which includes the male pronucleus (Naito *et al.*, 1988).

Table 2.2.6.1 The composition of fertilization medium BO-IVF (Nedambale *et al.*, 2006)

Constituent	**Concentration (mM)**
Caffeine	10
Heparin	10µg/ml
Glucose	13.9
$NaHCO_3$	37
$CaCl_2.2HO_2$	2.25
NaCl	112
Bovine serum albumin (BSA)	5mg/ml
BSA-FAF	6mg/ml
KCl	4.02
$NaH_2PO_4.H_2O$	0.83
$MgSO_4.7H_2O$	0.52
Na-pyruvate	1.25
Phenol red	10µg/ml
Antibiotic (100x)	10µl/ml

Electrical stimulation of ootids releases an electrical pulse to the ootid that mimics fertilization. It can also be known as artificial activation as it allows for the ootid to be activated to progress to the blastocyst stage without the presence of a sperm cell. Wang *et al.* (1998b) found that electrical stimulation had a higher ootid activation result when compared to another form of activation: the use of calcium ionophore. The activation procedure is undertaken in order to compare the activation of ootids with electrical stimulation and sperm penetration (Wang *et al.*, 1998b). During normal fertilization the cytoplasm of a matured ootids is activated to continue advancing in its development, electrical stimulation allows researchers to mimic this process (Whittingham, 1980; Yamauchi *et al.*, 1996). This is done in order to determine the quality of porcine ootids following maturation without being affected by *in vitro* fertilization with sperm and the incidence of polyspermy (Nagai, 1994; Liu & Moor, 1997).

Oocytes that have been removed from ovaries through the slicing method versus aspiration have an increased ability to cleave following activation (Liu & Moor, 1997). The maturation conditions of oocytes prior to activation and the subsequent effect on the oocyte is not fully understood and requires more study (Yamauchi *et al.*, 1996). Although it has been shown that over maturing of oocytes will

decrease the incidence of pronuclei formed following electrical stimulation (Borsuk, 1991; Naito & Toyoda, 1991; Funahashi *et al.*, 1993; Kikuchi *et al.*, 1995; Yamauchi *et al.*, 1996).

Electrical stimulation of ootids takes place in a buffered medium containing sugars such as mannitol, inositol or sorbitol; with mannitol being the sugar of choice for porcine oocytes (Liu & Moor, 1997; Sato *et al.*, 2005; Im *et al.*, 2007; Koo *et al.*, 2008; Kwon *et al.*, 2014). Ootids are placed in 300µl of electroporation medium that contains 0.3M mannitol in distilled water as well as 1mg/mL BSA and 5% TL-HEPES solution (Hagen *et al.*, 1991; Schoenbeck *et al.*, 1993; Yamauchi *et al.*, 1996; Wang *et al.*, 1998b). Isom *et al.* (2012) made use of a medium of 0.3M mannitol, 1mM $CaCl_2$ and 0.5mM HEPES solution. Electroactivation of porcine ootids is ineffective unless the electroporation medium contains Ca^{2+} and Mg^{2+} (Didion *et al.*, 1990; Sun *et al.*, 1992; Liu & Moor, 1997). Kwon *et al.* (2014) found that placing the oocytes in the mannitol solution for an exposure time of 1 minute prior to and 3 minutes post activation increased the rate of blastocyst formation (Eakin & Hadjantonakis, 2006). The ootids undergo a 5-10 second pulse at 4 $V.mm^{-1}$ AC and thereafter a single pulse at 120 $V.mm^{-1}$ DC for 30 µ seconds in a BTX Electro-Cell Manipulator 200 (Hagen *et al.*, 1991; Schoenbeck *et al.*, 1993; Yamauchi *et al.*, 1996; Wang *et al.*, 1998b; Isom *et al.*, 2012). Liu & Moor (1997) found that oocytes that undergo multiple pulses, specifically 3 pulses at 5 minutes apart, had superior activation and development.

Post electroporation the activated zygotes are placed in 50µl drops of culture medium placed in an environment of 5% CO_2 at 39°C for 12-15 hours (Schoenbeck *et al.*, 1993; Wang *et al.*, 1998b). This procedure may also be extended for use prior to *in vitro* fertilization where sperm cells are to be present.

Table 2.2.6.2 The composition of the ES media mannitol solution (Kwon *et al.*, 2014)

Constituent	**Concentration (mM)**
D-mannitol	0.26M
$MgCl_2$	0.1
$CaCl_2$	0.1
HEPES	0.5
BSA (bovine serum albumin)	0.05%

To check for nuclear activation of the fertilized and electrically stimulated zygotes, the zygotes will be suspended in paraffin oil on a coverslip and fixed with an acetic acid and ethanol mix. The ratio of the acetic acid and ethanol mix is to be 13:3 or 3:1 and thereafter stained with 1% aceto-orcein (Schoenbeck *et al.*, 1993; Yamauchi *et al.*, 1996; Agung *et al.*, 2013). Activated or fertilized embryos are identified when cleavage occurs or there is the presence of either one or two pronuclei (Yamauchi *et al.*, 1996). Those embryos that are checked for cleavage and pronuclei using staining were not able to develop further.

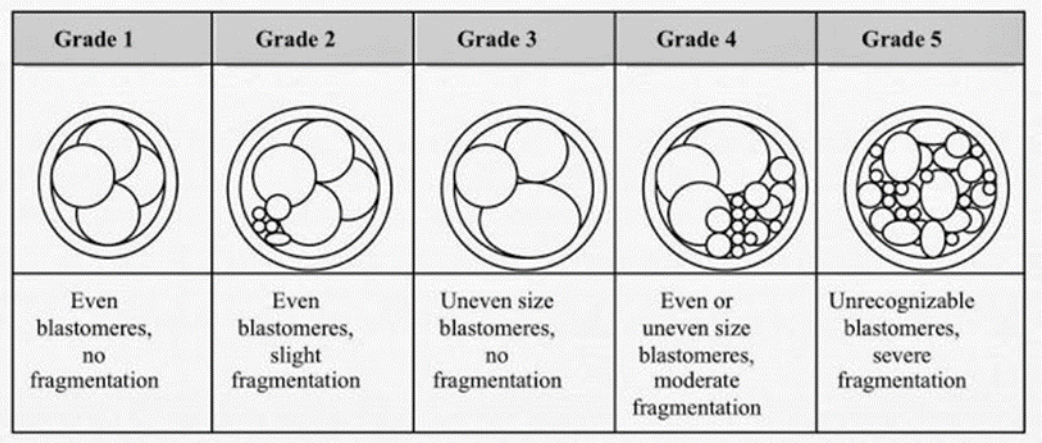

Figure 2.2.6.1 A grading system for embryo quality (Fields, 2019)

Embryos can be classified into five groups to describe the observable quality. Good quality embryos can be classed as Grade 1, medium quality embryos are categorized as Grade 2 and Grade 3, while poor quality embryos are categorized as Grade 4 and Grade 5 (Figure 2.2.6.1) (Demiray *et al.*, 2017; Fields, 2019). Embryos that arrest or that stop growth and start degrading are known as lysed embryos (Figure 2.2.6.2). Due to polyspermic embryos advancing to the blastocyst stage at the same rate as monospermic embryos it is difficult to separate and grade them independently (Han *et al.*, 1999a; b; Gil *et al.*, 2010). Isom *et al.* (2012) found that porcine embryos that undergo cleavage earlier than their counterparts have a higher incidence of advancing to the blastocyst stage (Booth *et al.*, 2007; Dang-Nguyen *et al.*, 2010).

Figure 2.2.6.2 The visual difference between a healthy and arrested embryo ('EmbryoScope: Arrested Embryo Compared to Healthy Embryo', 2017)

2.2.7 *In vitro* culture (IVC)

The medium used for *in vitro* culture (IVC) can influence the efficiency of fertilization following IVF (Gil *et al.*, 2010). Although, the *in vitro* culture of porcine embryos is inferior to the *in vivo* production of embryos (Gil *et al.*, 2010). IVC has improved over time as new techniques have been formed and processes, such as using a culture dish instead of a tube, will increase our understanding on IVC as well as our laboratory methods (Jones *et al.*, 1982; Marrs *et al.*, 1983, 1984). The use of IVC

media is still under scrutiny as multiple media have been tested but no one medium has been deemed ideal (Edwards, 1981; Trounson, 1983; Marrs *et al.*, 1984).

Centrifugation of embryos, at 10000-15000 xg for 10-20 minutes, occurring 10-16 hours after fertilization can allow for the selection of monospermic zygotes as the pronuclei can be visualized following this process without negatively affecting the chance of advancing to the blastocyst stage (Gil *et al.*, 2010). After 24 hours of co-culture the fertilized ootids are to be washed in HEPES-Synthetic oviduct fluid medium (SOFM) twice and placed into 40µl of culture medium (Baldassarre *et al.*, 1996).

The current most successful media used for IVC are NCSU 23 and NCSU 37 (Petters & Wells, 1993; Gil *et al.*, 2010). Studies suggest that the NCSU media be supplemented with lactate and pyruvate for 48-72 hours and thereafter be supplemented with glucose, as 2-4 cell embryos show to have less glucose requirements than more developed embryos (Gandhi *et al.*, 2001; Gil *et al.*, 2010). This shows superior rates of blastocyst development and total cell number when compared to media that contain glucose continuously (Abeydeera, 2002; Kikuchi *et al.*, 2002; Gil *et al.*, 2010). The culture medium should lack caffeine following an IVF medium which contains caffeine, this allows for an increased chance of normal fertilization as it can control the binding and penetration of sperm to the oocyte (Funahashi & Romar, 2004). However the use of caffeine or lack thereof during IVF and IVC respectively does not influence the rate of cleavage and development to the blastocyst stage (Funahashi & Romar, 2004).

The embryos are placed in the culture medium, NCSU 23, are supplemented with 4 mg/ml BSA covered with mineral oil, and incubated at 39°C with 5% CO_2, 7% O_2 and 88% N_2 for 7 days (Baldassarre *et al.*, 1996; Wang *et al.*, 1998b; De Macedo *et al.*, 2019). Yoshioka *et al.* (2002, 2008) found that an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 is the optimal for embryo culture. Other studies have shown that low O_2 concentrations are best for the culture environment while others suggest that an environment of primarily O_2 is superior (Machaty *et al.*, 1998; Karja *et al.*, 2004; Kitagawa *et al.*, 2004; Gil *et al.*, 2010). Likewise, the oxygen levels during culture may be influenced by the embryo type, with morula stages preferring 20% O_2 and more developed embryos preferring 5% O_2 (Abeydeera, 2002; Booth *et al.*, 2005; Gil *et al.*, 2010). The embryos are placed in fresh media every 48 hours, but can be checked for cleavage at the initial 48 hours post fertilization, and then further checked for blastocysts 7 days post fertilization (Figure 2.2.7.1) (Baldassarre *et al.*, 1996; Wang *et al.*, 1998b; De Macedo *et al.*, 2019).

A blastocyst can be defined as an embryo with clear blastocoels present (Agung *et al.*, 2013). It has been shown that most porcine embryos reach the blastocyst stage by day 5 but are checked on day 7 to make allowances for those embryos that were delayed in growth (Pattern, 1948; Abeydeera & Day, 1997; Senger, 1997; Hao *et al.*, 2004; Dang-Nguyen *et al.*, 2010; Zhao *et al.*, 2010; Jeon *et al.*, 2011; Isom *et al.*, 2012).

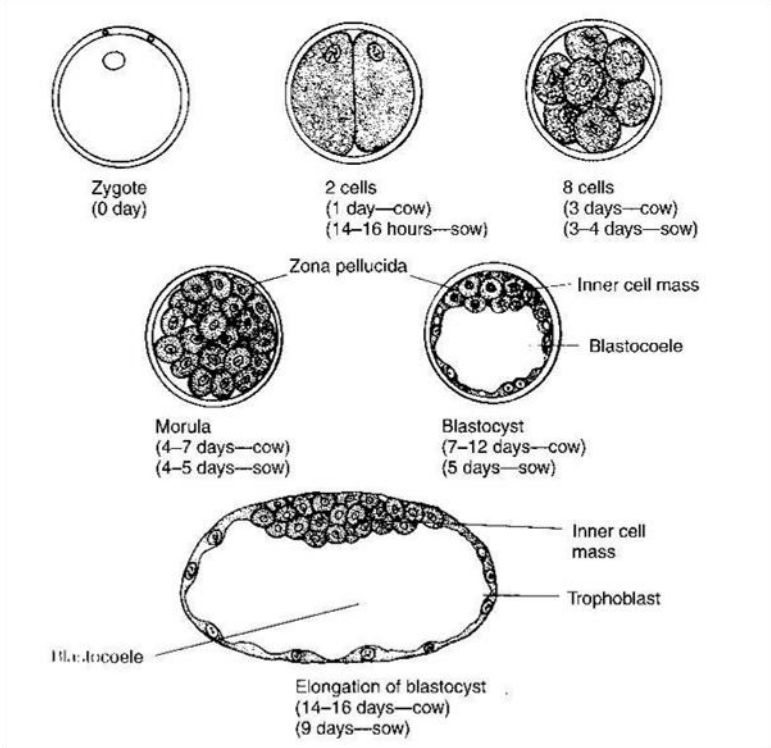

Figure 2.2.7.1 Embryonic development at certain times after fertilization (Bearden *et al.*, 2004)

2.3 Conclusion

It can thus be seen throughout literature that *in vitro* studies within the porcine industry has a large knowledge gap that needs to be identified. In South Africa especially the porcine industry has room to grow in order to be on par with other countries. The use of assisted reproductive technologies can be used to improve the industry by working at the root of the problem, with the germplasm. Germplasm is able to be imported and exported between countries where live animals are not, this allows for the opportunity to transport genetic material. This genetic material from other countries may not normally be made available but through technologies such as oocyte removal and cryopreservation of embryos it is made possible. This will allow for new genetic availability for local populations as well as decreasing the risk of disease transmission and is more cost effective than a live animal. Gametes and embryos imported from genetically superior animals may then be implanted into a female within the environment the new animals may be raised in. This will then give the progeny maternal passive immunity. The progeny will then be less susceptible to the environment they were born into rather than being transported to post farrowing. This all may be possible but the reproductive technologies have not yet been perfected and not every gamete or embryo that is used will develop into a healthy piglet. Therefore more research and physical studies are needed to find the ideal conditions to retrieve, mature, fertilize and culture porcine oocytes. Each new development is a step forward in advancing the industry of porcine reproduction in South Africa which could continue the growth within the pork producing industry that will make the country more self-sustainable.

Chapter 3: Materials and Methods

3.1 Introduction

In the study different preselected reproductive technologies were used at each stage of the experiment; these would include oocyte retrieval, IVM, ES, IVF and IVC. This was done to observe which of the reproductive technologies used are superior for porcine oocytes to improve upon the current shortfalls found within the South African pork industry.

The study is aimed to investigate the oocyte retrieval, maturation, fertilization and culturing effect on porcine embryos produced *in vitro*. The specific objectives were to: (1) To determine the effect of retrieval techniques (slicing and aspiration) on the quality of porcine oocytes. (2) To compare three different *in vitro* maturation media (NCSU 37, TCM 199 and follicular fluid + FSH & LH) on porcine oocyte maturation rate. (3) To compare electrical stimulation and frozen-thawed semen on fertilization rates following IVF. (4) To compare two different *in vitro* culture media (cNCSU 37 and cpFF) on the cleavage rates of porcine embryos.

Ethical clearance was obtained from Agricultural Research Council- Animal Production Campus (ARC-AP) for use in the Germplasm, Conservation, Reproduction and Biotechnologies (GCRB) laboratories, APAEC [2019/24]. Ethical clearance was likewise obtained from the University of Pretoria for laboratory work at the ARC-AP, NAS215/2020.

3.2 Materials

3.2.1 Chemicals

The chemicals used in the study were obtained from Sigma-Aldrich prior to the commencement of the study. Chemicals or apparatus not obtained from Sigma-Aldrich were indicated otherwise.

Table 3.2.1 The chemicals used by the researcher through the entirety of the study

Materials	**Amount**
DPBS	500ml
1% PVA	250G
Antibiotic Antimycotic	20ml
Phenol Red	100ml
M199 (with Earles salt)	450ml
FBS	500ml
NaOH	3ml (optional)
Ultrapure water	1000ml
100% ethanol	350ml
Stock TCM 199	500ml
Na-Pyruvate	100G

D-Glucose	100G
Cysteine	100G
EGF	2MG
NaCl	500G
$NaHCO_3$	1 kg
KCl	250G
KH_2PO_4	100G
$MgSO_4.7H_2O$	500G
$CaCl_2.2H_2O$	500G
Glucose	100G
Glutamine	100G
Sorbitol	1kg
Insulin-Transferrin-Sodium	1VL
Penicillin-G-Potassium salt	10Mμ
Streptomycin	10ml
PFF	50ml
FSH	10UG
LH	50UG
$MgCl_2.6H_2O$	100G
$NaH_2PO_4.H_2O$	500G
Gentamicin	10ml
$CaCl_2.H_2O$	500G
Caffeine	100G
BSA	10G
Mannitol	500G
5% TL-HEPES	20ml

Table 3.2.2 The equipment and specific chemicals used for oocyte recovery and grading per replicate

Materials	**Amount**
Thermal flask	1
Thermometer	1
Saline solution	1
Water bath	1
Paper towel	1 industrial roll
Stainless steel kidney dish	1

50ml tube	3
mDPBS	10ml
Permanent marker	1
Petri dish	1
Scalpel	1
Syringe	1
18G needle	1
Handheld pin	1
Gloves	1 box
Sharps bin	1
Pasteur Pipette	1
Dissecting microscope	1
Falcon 1008 petri dishes	6
Petri dish	2
Pipette	1
Pipette tip	2
Ruler (30cm)	1
Scissors	1
mDPBS	9ml
M199	9ml

Table 3.2.3 The equipment and specific chemicals used for *in vitro* maturation per replicate

Materials	**Amount**
0.20µl single use filter	6
10ml syringe	6
50ml tubes	26
Gloves	1
Falcon 1008 petri dishes	4
Thermo Scientific 4 well multidish	1
Eppendorf tubes	4
Pipette	1
Pipette tips	8
TCM 199	10.5ml
NCSU 37	500µl
mPFF	500µl
Mineral oil	1000µl

Olympus Oosight Nuclear Transfer microscope	1
Dissecting microscope	1
Lasec Vortex Mixer	1
Hermle Z300 K centrifuge	1

Table 3.2.4 The equipment and specific chemicals used for *in vitro* fertilization, electrical stimulation and *in vitro* culture per replicate

Materials	**Amount**
0.20µl single use filter	5
20ml syringe	5
50ml tubes	8
Falcon 1008 petri dishes	4
Falcon 3801	6
Eppendorf tubes	2
Pipette	3
Pipette tips	10
TCM 199	10ml
ES media	4ml
IVF media	10ml
cNCSU 37	1ml
cpFF	1ml
Mineral oil	18ml
Dissecting microscope	1
BTX ECM 2001 Electro Cell Manipulator	1
BTX Model 450 Microslide 0.5mm	2
Liquid nitrogen tank	1
Frozen-thawed semen straws	1
Forceps	1
Minitube Model Cito, 38°C	1
Thermometer	1
Hermle Z300 K centrifuge	1
15ml tubes	1
Pasteur pipette	1
Tissue paper	1 box
Scissors	1
Lasec Vortex Mixer	1

CASA microscope	1
SCA® software	1
Microscope slide	1
Cover slip	1

3.3 Methods

3.3.1 Study site

The study was conducted at the Germplasm Conservation and Reproductive Biotechnologies (GCRB) laboratories of the Agricultural Research Council-Animal Production (ARC-AP) in Irene, Pretoria. The ARC-AP is situated at 25°55' S and 28°12' E and is found on the Highveld at an altitude of 125m above sea level (Webb *et al.*, 2004; Lekola, 2015).

3.3.2 Transportation of porcine ovaries

The ovarian tissue was obtained from prepubescent gilts of unknown breeds three times a week on a Monday, Wednesday and Friday from the Tiger Brands abattoir in Olifantsfontein, Johannesburg. The ovaries were placed in a thermos flask (Cole-Parmer, West-Germany) and covered with 0.9% NaCl saline solution (SABAX, South Africa) at 39°C, measured with a thermometer (Alla France, France), and transported from the abattoir to the laboratory within one hour after slaughter.

Figure 3.3.2.1 The flask used for transportation of porcine ovaries (left) with saline water being warmed (right)

Upon arrival the saline solution was drained and the ovaries were sprayed with 70% ethanol and placed in a kidney dish covered with fresh, warmed saline solution. The kidney dish was placed in a water bath (MacDonald Adams & Company, South Africa) at 39°C for the duration of oocyte removal.

Figure 3.3.2.2 Porcine ovaries placed in a kidney dish in a water bath

3.3.3 Aspiration of follicles

The ovaries were retrieved one at a time from the kidney dish and patted dry using a paper towel. An 18 gauge needle (Neomedic, United Kingdom) was attached to a 10ml syringe (Surgi Plus, China) and inserted into the ovary surface to remove the oocytes from medium sized follicles (3-6mm diameter). The follicular fluid collected inside the syringe was placed into a 50ml tube (Deltalab, Spain) containing 5ml of modified Dulbecco Phosphate Buffered Saline (mDPBS) (Pan Biotech, Germany) until further evaluation.

Figure 3.3.3.1 Aspiration of the porcine ovary

3.3.4 Slicing of ovaries

During the slicing technique the ovaries were placed in a petri dish (Thermo Scientific, South Africa) containing 5ml of mDPBS. A handheld pin (Lasec, South Africa) was used to hold the ovary in place while a size 22 surgical blade (Paramount Surgimed Ltd, Germany) cut through the entire ovary multiple times. The remaining fluid inside the petri dish was poured into a 50ml tube until needed for further evaluation. The tube was kept in the water bath in order for a pellet and supernatant to form. The supernatant was then gently removed using a Pasteur pipette (LP Italiana, Italy) without disturbing the pellet. More mDPBS was then added into the tube and repeated until the remaining fluid was clear enough for grading.

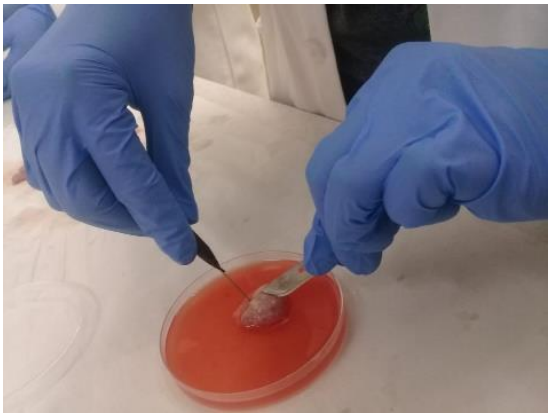

Figure 3.3.4.1 The slicing of a porcine ovary

3.3.5 Preparation of the oocytes prior to IVM

Six Falcon 1008 dishes (Becton Dickinson, USA) were prepared with three dishes containing 3ml of mDPBS each and the remaining three dishes containing 3ml of M199 supplemented with 10% of FBS. The dishes were placed in the incubator (Thermo Electron Corporation, USA) at 38.5°C prior to oocyte washing. The oocytes were then washed three times with mDPBS and thereafter washed a further three times with M199 (Figure 3.3.5.1) supplemented with 10% of FBS with the aid of a handheld pipette (Rainin, USA). Prior to IVM, the oocytes were graded under the stereo microscope (Olympus, Japan and Taiwan) and classified into grades A, B or C (Marrs *et al.*, 1984; Hamano & Kuwayama, 1993; Khandoker *et al.*, 2001; Lekola, 2015; Kumar *et al.*, 2016). Grade C oocytes were discarded while grade A and B oocytes were selected for maturation.

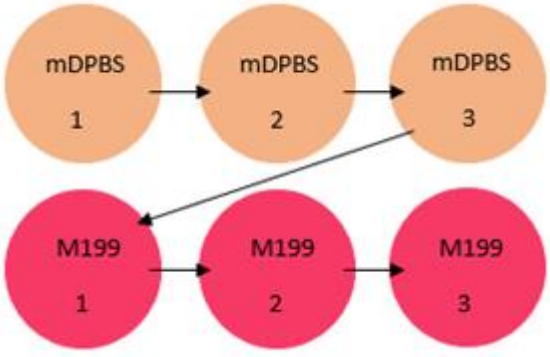

Figure 3.3.5.1 A visual aid on the oocyte washing procedure

3.3.6 Preparation of IVM, IVF, ES and IVC media

In brief, the IVM, ES, IVF and IVC media was prepared under the lamina flow using glass beakers, while the chemicals were weighed out on the scale (Denver Instrument, Germany). The prepared media was filtered through 0.20µl single use filters (Gema Medical S.L., Spain) and placed in labelled 50ml tubes. The lids of the tubes were closed completely and sealed using parafilm (Lasec,

USA) and placed in the fridge (5°C). The solution was used within two weeks and thereafter discarded, with fresh media then being prepared for use.

3.3.7 IVM procedure

A total of 500µl of each of the IVM media was placed in a four-well dish (Thermo Scientific, USA), with each media having its own separate well (Figure 3.3.7.1). The wells were then covered with 250µl of mineral oil, to prevent evaporation, and labelled to record which media was being used. The grade A and B oocytes were then placed in the prewarmed media and incubated at 38.5°C with 5% CO_2 for 44 hours.

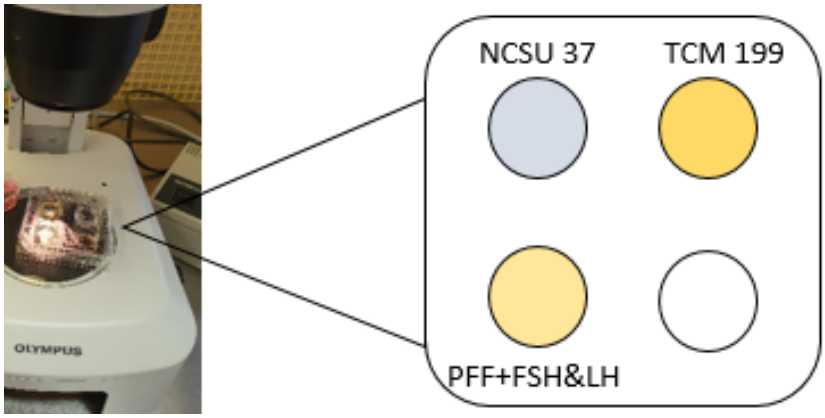

Figure 3.3.7.1 A visual aid, including a reference image, on the IVM procedure

3.3.8 Evaluation of ootids polar body extrusion

The ootids were removed from the IVM media at the end of the IVM period of 44 hours (Yamauchi *et al.*, 1996; Agung *et al.*, 2013). Matured ootids were then placed in an eppendorf tube with 200µl of prewarmed M199 and then vortexed (Benchmark Scientific Inc., Taiwan) for three minutes (Zeringue *et al.*, 2005). Following this process, the denuded ootids were then transferred to a mini petri dish containing 3ml of M199. The ootids polar body extrusion was evaluated with the aid of Oosight Imaging System (Hamilton Thorne) connected to an inverted research microscope (Olympus, Japan and Philippines) and recorded per treatment.

3.3.9 Semen quality, thawing of boar semen straws and centrifuging

Frozen semen straws used for IVF were removed from a liquid nitrogen tank (-196°C, Custom Biogenic Systems, USA) using forceps. The frozen straw was held in the air for 10 seconds, then plunged into a warm (37°C) water container (Minitube, Germany) for 1 minute. The straw was then patted dry using paper towels before one side of the semen straw was cut and poured into 8ml of prewarmed IVF media. The other side of the straw was cut thereafter to ensure all the semen was released into the tube. The IVF-semen solution was centrifuged (Hermle Labortechnik, Germany) at 2000rpm for 2 minutes at 36°C. The supernatant was removed with a pasteur pipette and replaced with another 8ml of IVF media. The solution was then centrifuged once again and the supernatant removed

without disturbing the pellet. The sperm pellet was then diluted with more IVF media and placed on a warming plate (Kunz Instruments, Denmark).

Prior to IVF taking place the CASA-SCA® system was used to determine sperm motility and velocity traits. The semen sample was taken from the same colour straw as those used in the study. This indicates that the spermatozoa used throughout was obtained from one ejaculate from a specific boar. 5µl of the frozen-thawed semen sample was placed on to a previously warmed microscope slide (~76 x 26 x 1mm, Waldemar-Knittel, Germany) and a warmed cover slip (22 x 22mm, Waldemar-Knittel, Germany) was placed on top of the droplet. The slide was placed on a warming plate at 37°C (Kunz Instruments, Denmark) prior to the placement of the droplet and cover slip. The slide was then placed onto the CASA-SCA® system (Microptic, Spain) and two to three fields were captured for assessment. The capturing took place at 10x magnification (Nikon, Japan) in order to track each individual sperm and the SCA® software was used to record the images. The fields captured were then examined for possible debris to be removed manually so as to decrease the possibility of capturing unclear tracks.

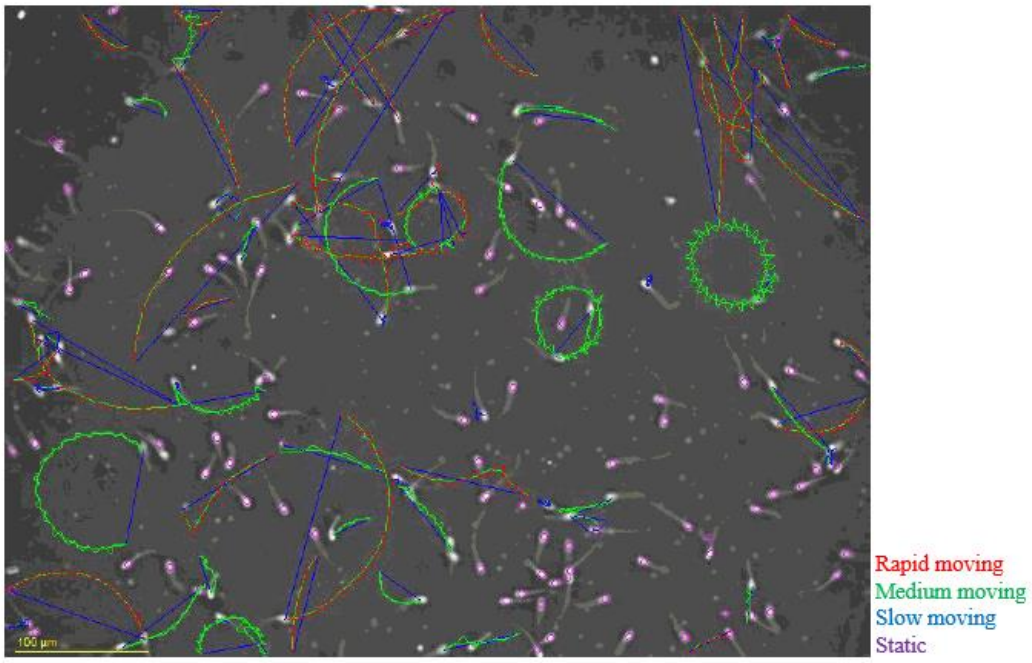

Figure 3.3.9.1 The CASA-SCA® system showing the different pattern of sperm motility traits from a sample of thawed semen used in the study

3.3.10 IVF of matured ootids

The matured ootids were washed five times in a previously prepared Falcon 3801 dish (Becton Dickinson, USA) containing 100µl drops of the IVF media covered with 3ml of mineral oil. The ootids were allocated into a dish previously prepared with seven 50µl drops of the IVF media covered with 3ml of mineral oil (Figure 3.3.10.1). A total of 50µl of the semen/ sperm was added to each 50µl drop,

with a final volume of 100µl, containing the selected ootids. The IVF dish was then placed into an incubator for 24 hours at 5% CO_2 at 38.5°C.

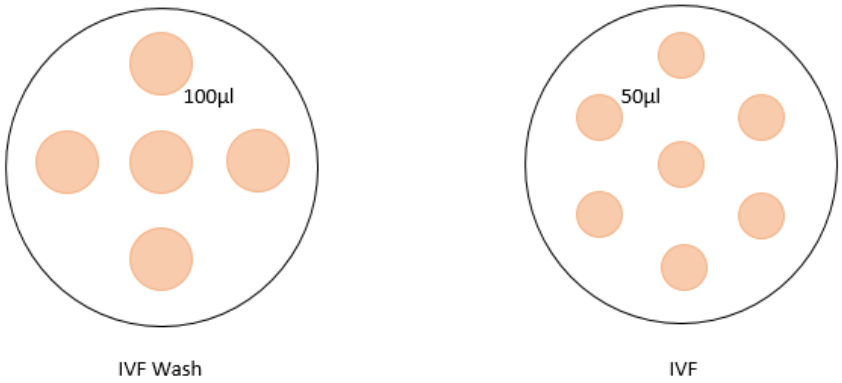

Figure 3.3.10.1 A visual aid on the IVF procedure

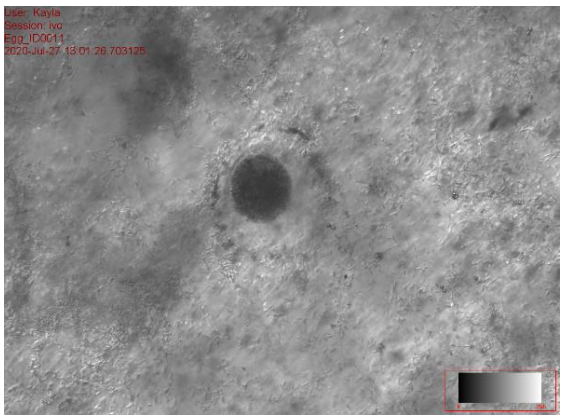

Figure 3.3.10.2 Sperm cells surrounding an ootid (4x)

3.3.11 ES of matured ootids

Matured oocytes were washed five times in previously prepared 100µl drops of ES media covered with 3ml of mineral oil (protocol same as Figure 3.3.10.1). The matured oocytes were then placed in the ES media for 1 minute prior to and 3 minutes post stimulation. The ootids were placed between the 0.5mm gap in a BTX Model 450 dish (Harvard Apparatus Inc., USA) (Figure 3.3.11.1). The dish was then connected to the BTX ECM 2001 Electro Cell Manipulator (BTX Harvard Apparatus, USA) and given 4 V/mm AC for 10 seconds and thereafter 2 pulses of 60 V/mm DC for 60 µseconds; with two repeats.

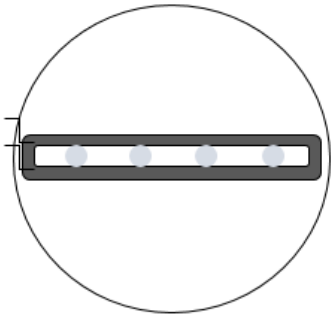

Figure 3.3.11.1 A visual aid for the placement of media drops containing ootids in the BTX Model 450 dish

3.3.12 IVC of embryos

The IVC of embryos was done immediately following ES and 24 hours post IVF. The zygotes that were used for IVF get placed, 24 hours post IVF, in an eppendorf tube (Simport, Canada) and vortexed for 1 minute and 30 seconds. Thereafter the procedure is the same for both ES and IVF. The zygotes from the ES and IVF treatments were divided into two groups each and allocated to the IVC media, cNCSU 37 and cpFF, so that four dishes were present after IVC. The zygotes were washed 3 times in M199 and thereafter washed 5 times in previously prepared 100µl drops, covered with 3ml of mineral oil, of either cNCSU 37 or cpFF media (protocol same as Figure 3.3.10.1). The washed zygotes were then placed in 50µl of the corresponding prepared IVC media covered with 3ml of mineral oil. The dishes were then immediately put into a modulator incubator chamber (Billups-Rothenberg, USA) with 90% CO_2 at 38.5°C for 48 hours.

3.3.13 Evaluation of embryo cleavage

Two days post IVC the embryos were removed from the incubator and modulator incubator chamber and checked under the dissection microscope for signs of cleavage. The embryos were visually judged and grouped, according to cleavage cell number, into 1 cell, 2-4 cell, 8+ cell and lysed. The cleavage percentage could thereafter be determined.

3.3.14 Evaluation of blastocyst quality

On day 5 and day 7 all the embryos were examined using the microscope to determine whether they had advanced to the morula or blastocyst stage respectively. The morula and blastocyst percentage could then be calculated and the embryos were checked for viability for further procedures. The embryos were either destroyed by using standard procedures or suspended using an acetic acid & ethanol solution or a formaldehyde solution.

3.4 Data editing

Data analysis for objective one and two used the General Linear Model (GLM) procedure, including ANOVA and the t-Test (Fisher's LSD); for significant differences between groups. Univariate statistical analysis was used which included basic statistical measures, tests for location, tests for normality, quantiles, extreme observations, the normal probability plot and variable moments. Variable moments include the statistical parameters: mean, variance, standard deviation and skewness.

3.5 Statistical analysis

Data collected was analysed using an analysis of variance (ANOVA) test and a Shapiro-Wilk's test was used to test for deviations from normality (Shapiro & Wilk, 1965). Statistical significance of the researchers t-LSDs (least significant differences) were evaluated at a $P < 0.05$; where a 5% significance level was used for the comparison of treatment means. The data was analysed using the statistical software SAS version 9.4 (SAS, 1999).

Chapter 4: Results

Porcine *in vitro* studies are challenging to complete due to pig oocytes being difficult to work with. Nevertheless every study that is completed allows us to expand our knowledge on how to handle and develop porcine embryos *in vitro*. Four objectives were undertaken in this study and followed the progression of the porcine embryo from the immature oocyte to the cleaved zygote.

4.1 The comparison of oocyte removal techniques

The first objective addressed the techniques used in oocyte removal. Experiment one focused on the comparison of two techniques, aspiration and slicing. The aspiration technique punctured the follicles with a needle while the slicing method cut the follicles to release the fluid. The oocytes, after the respective removal techniques, were visually assessed for quantity and quality using the microscope. It was found in this study that there was significant differences ($P<0.05$) between the techniques, with the slicing method being found to yield higher amounts and better quality of oocytes than the aspiration method (Table 4.1).

Table 4.1 The means and standard error/deviations of the comparison of oocyte removal techniques

Method	**Number of ovaries**	**Oocyte classification (grade)**			
		A	**B**	**C**	**Total**
Aspiration	20	19.6±6.4[a]	78.8±22.6[a]	95.4±41.9[a]	193.8±53.9[a]
Slicing	20	45.6±15[b]	241.2±102.5[b]	201.8±86.7[b]	488.6±159.2[b]

[ab] - Means with different superscript letters in the same column differed ($P\leq0.05$)

Over the course of the first objective a total amount of 682.4 oocytes were collected from porcine ovaries and graded. Of the total oocytes collected, 28.4% of those were retrieved using the aspiration method while the remaining 71.6% were retrieved with the slicing method (Figure 4.1). Thereafter the quality of the oocytes were assessed according to the guidelines shown by Kumar *et al.* (2016). Grades A and B were selected for further work while grade C was discarded, therefore the grouping of grades A and B were defined as good quality oocytes for the duration of this study. Good quality oocytes accounted for 50.8% of the total oocytes collected for aspiration while 58.7% accounted for those collected using the slicing method. This lead the researcher to conclude that the slicing method was superior in this study for the quality of oocytes collected along with the previously mentioned quantity.

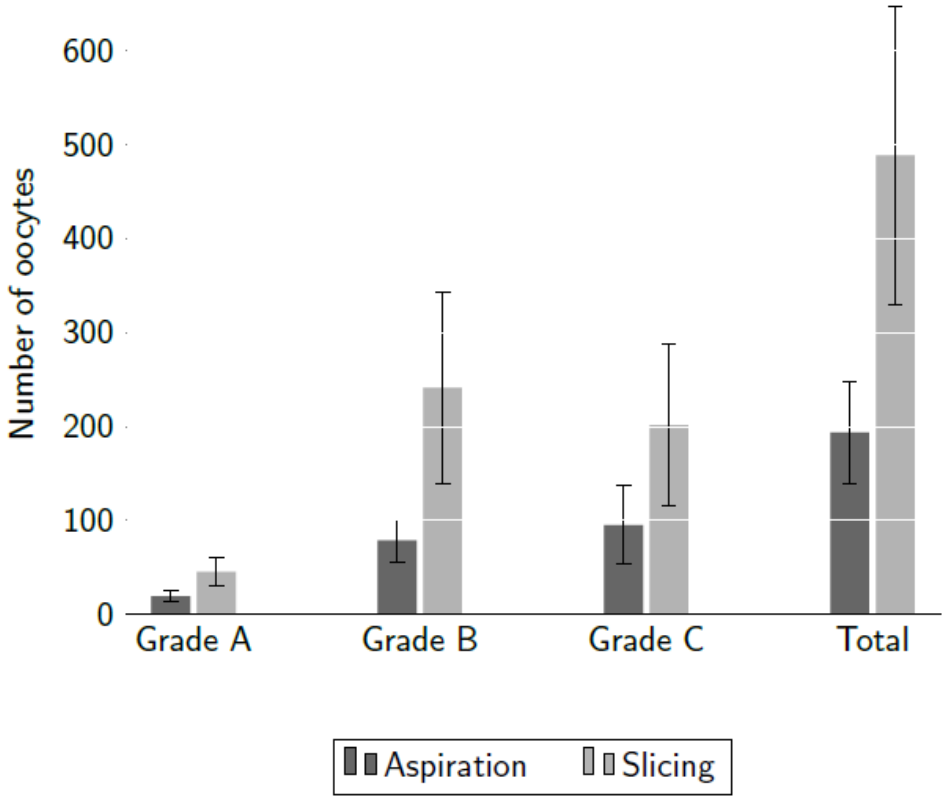

Figure 4.1 The comparison of oocyte removal techniques

4.2 The comparison of maturation media on oocytes polar body extrusion

The following objective undertaken in the study was *in vitro* maturation of porcine oocytes. The grade A and B oocytes were taken from the previous objective and randomly placed into one of three media. The media chosen for use in this study was NCSU 37, TCM 199 and mpFF. Oocytes polar body extrusion was evaluated following the completion of oocyte maturation. No significant differences were found between the treatments (P>0.05) (Table 4.2).

Table 4.2 The means and standard error/deviations of the comparison of maturation media on oocytes polar body extrusion

Media	Number of oocytes	Oocytes observed	Polar body status
NCSU 37	159	26.5±18.2	6.7±9.7
TCM 199	159	26.5±18.2	22.3±29.03
mpFF	158	26.3±18.3	19.4±19.8

[abc] - Means with different superscript letters in the same column differed (P≤0.05)
NCSU- North Carolina State University
TCM- Tissue Culture Medium
mpFF- Porcine follicular fluid modified for *in vitro* maturation

The polar body extrusion was found to be 25.3% for NCSU 37, which upon initial inspection has the lowest percentage. TCM 199 had a polar body extrusion rate of 84.2% and mpFF had a rate of 73.8% (Figure 4.2). This did not allow the researcher to make an informed choice of which medium was superior. For further work for the duration of the study the researcher chose to continue with mpFF as a maturation medium.

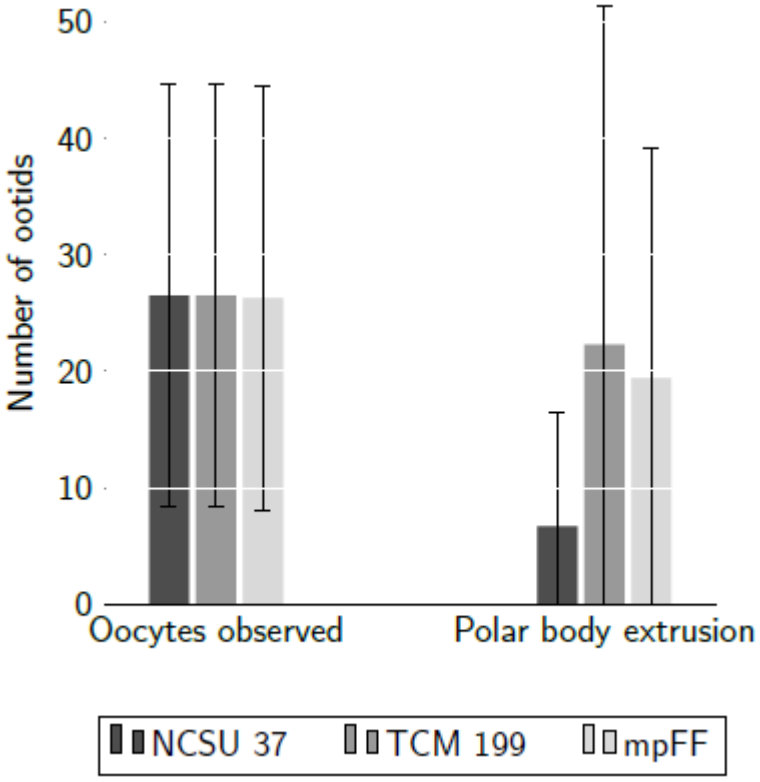

Figure 4.2 The comparison of maturation media on oocytes polar body extrusion

4.3 The comparison of electrical stimulation on oocytes and frozen-thawed semen on fertilization rates following IVF and the comparison of cell cleavage rates following IVC in two different culture media

The third and fourth objectives were undertaken simultaneously. The *in vitro* fertilization protocol included the use of frozen-thawed spermatozoa and electrical stimulation. The *in vitro* culture part of the study tested two media, cNCSU 37 and cpFF. The zygotes following IVF, ES and IVC were scrutinized for cleavage, single cells and lysed cells. Significant differences were found between the frozen-thawed spermatozoa and electrical stimulation; as well as between the two IVC media cNCSU 37 and cpFF ($P<0.05$) (Table 4.3).

Table 4.3 The means and standard error/deviations of the comparison of electrical stimulation on oocytes and frozen-thawed semen on fertilization rates following IVF and the comparison of cell cleavage rates following IVC in two different culture media

Method	No. oocytes (IVF)	IVC medium	No. oocytes (IVC)	Lys	1 cell	2-4 cell	Total cleaved
ES	234	cNCSU37	120	19.3±15.3^{a}	38.9±25.1	41.8±19.7^{ab}	80.7±15.3^{a}
		cpFF	106	47.6±37.9^{ab}	27.1±21.7	25.4±24.3^{bc}	52.4±37.8^{bc}
FT	243	cNCSU37	112	26.2±21.9^{bc}	27.3±12.4	46.5±18.2^{a}	73.8±21.9^{ab}
		cpFF	103	56.9±38.9^{c}	31.2±29.6	11.8±11.9^{c}	43.1±38.9^{c}

^{abc} - Means with different superscript letters in the same column differed (P≤0.05)
ES- Electrical stimulation
FT- Frozen-thawed spermatozoa
IVF- *In vitro* fertilization
IVC- *In vitro* culture

The total number of cleaved zygotes for frozen-thawed IVF was 48.1% and was 56.8% for ES (Figure 4.3.1 and 4.3.2 respectively). These results lead the researcher to believe that ES had a greater influence on the cleavage rate of embryos than IVF. When observing the cleavage rate for the IVC media, the two IVF protocols had to be combined depending on which media was used. The total zygote cleavage for the cNCSU 37 media was 66.6% while the cleavage rate for cpFF was 45.7% (Figure 4.3.1 and 4.3.2). It was then concluded that the cNCSU 37 media had a greater effect on cleavage rate than cpFF. Therefore the oocytes that underwent ES during the IVF stage and then placed in cNCSU 37 during IVC performed the best.

In this study there was a 0% blastocyst rate, this is indicative that both the media selected by the researcher may have been poor choices for IVC of porcine embryos.

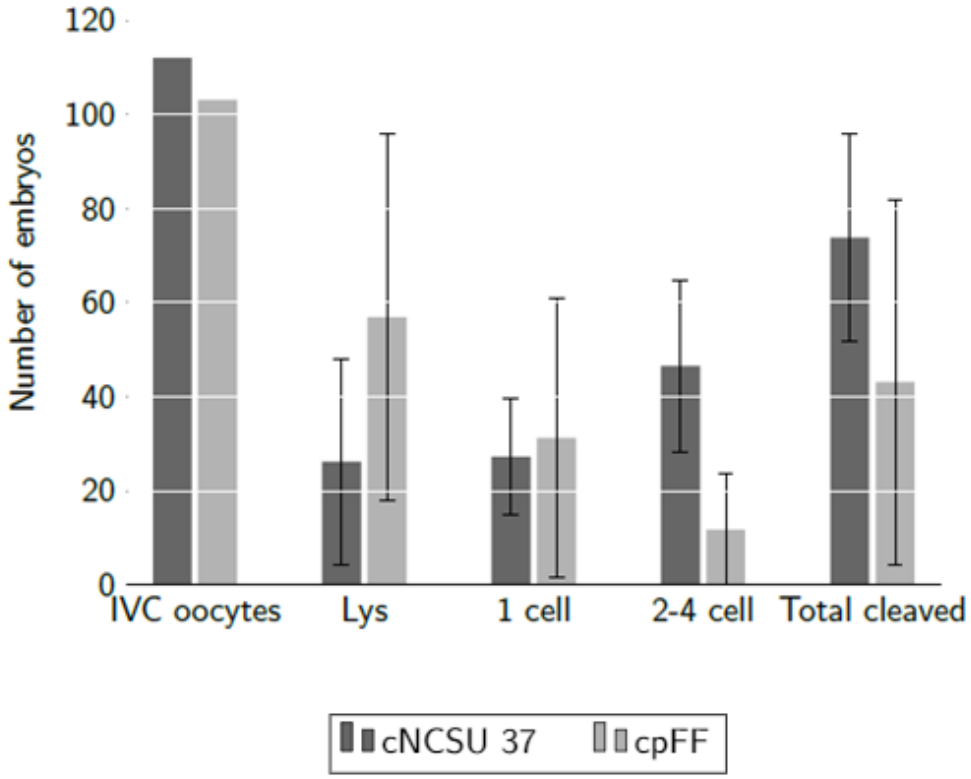

Figure 4.3.1 The comparison of two different culture media on cell cleavage rates following fertilization with frozen-thawed semen

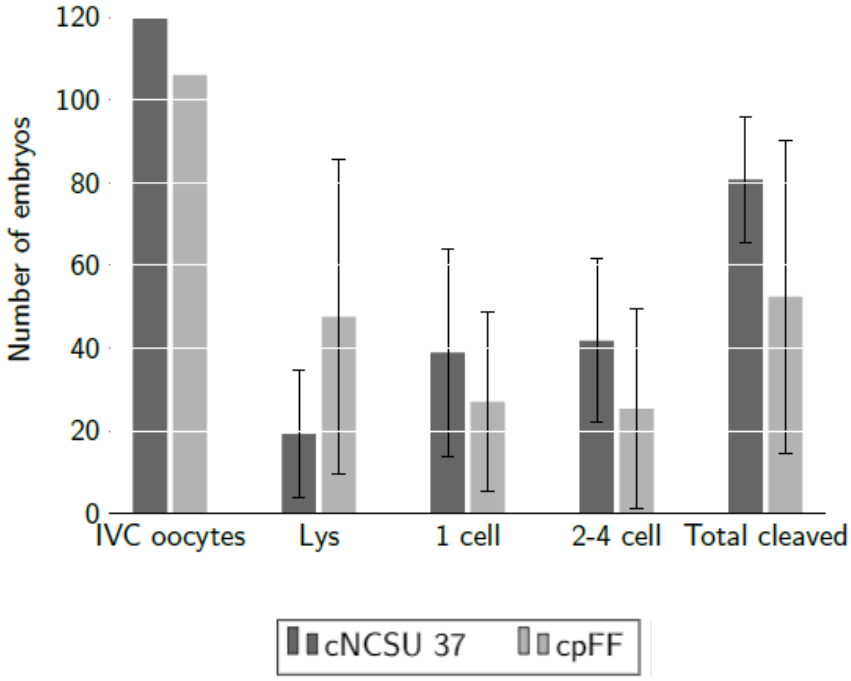

Figure 4.3.2 The comparison of two different culture media on cell cleavage rates following electrical stimulation

Chapter 5: Discussion

Work on porcine gametes, especially oocytes, is difficult but with each study we work towards improving our knowledge, bringing us one step closer to near perfect *in vitro* conditions. Improving the reproductive technologies in the porcine industry will allow for growth to occur in multiple countries. In South Africa the pork industry is severely underdeveloped compared to overseas counterparts (Robinson, 2018). If movement of high-quality porcine gametes (semen, oocytes and embryos) can occur between distant geographical locations then access could be gained to better genetics that would otherwise be unattainable. Mattioli *et al.* (1989) was the first researcher to produce viable embryos from IVM and IVF of porcine oocytes. These results that were produced paved the way for future researchers when using, and improving, reproductive technologies for porcine oocytes.

5.1 The comparison of oocyte removal techniques

This study was undertaken to determine the effect of retrieval techniques (slicing and aspiration) on the quality of porcine oocytes. The researcher hypothesized that the aspiration technique of removal would be found to be superior to slicing in terms of the quantity and quality of oocytes removed. The purpose of the first objective in this study was to collect a large quantity of high-quality oocytes; once this was achieved then the study was allowed to continue.

High-quality oocytes were defined, for this study, as grade A and B oocytes (Figure 5.1.1 and Figure 5.1.2 respectively). Therefore oocytes with 3-6 layers of compacted, multilayered cumulus cells and a homogeneous ooplasm were selected as high quality and used in further work with other reproductive technologies. Oocytes that have 0-2 layers of cumulus cells and partially denuded or completely denuded were defined as grade C (Figure 5.1.3) in this study and discarded according to the laboratory standard (Kumar *et al.*, 2016; Demiray *et al.*, 2017).

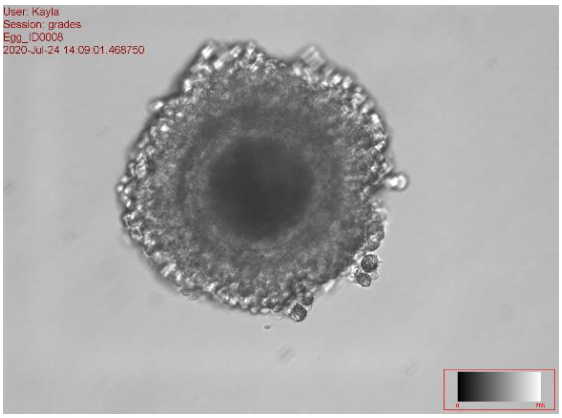

Figure 5.1.1 An example of a grade A oocyte (20x)

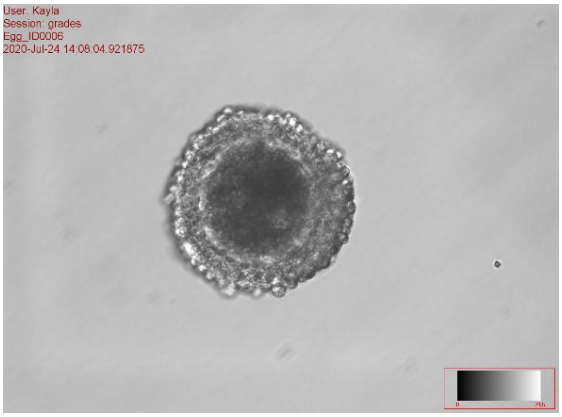

Figure 5.1.2 An example of a grade B oocyte (20x)

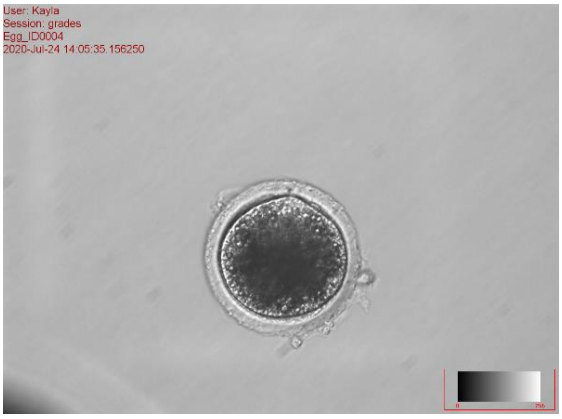

Figure 5.1.3 An example of a grade C oocyte (20x)

The results of this study show that there were significant differences ($P<0.05$) between the oocyte removal techniques (Table 4.1). Of the 682.4 oocytes collected over the entirety of the initial study, only 28.4% of those were collected using the aspiration method (Figure 4.1). The majority of oocytes collected were obtained through the slicing method, with 71.6% of the 682.4 oocytes collected using this method (Figure 4.1). This allows the researcher to deduce that the slicing method provides the highest amount of oocytes during collection when compared to aspiration. Marques *et al.* (2015) found similar results, showing that the slicing method produced a higher amount of oocytes than the aspiration method. These findings discredit the quantity portion of the hypothesis drawn up by the researcher.

While quantity is important for oocyte collection, quality is just as important. A high amount of low-quality oocytes could be collected, and while it may seem successful just due to the sheer numbers the collected oocytes may not be workable. This may occur in the opposite situation, a collection could yield few high quality oocytes that could all progress through the reproductive technology stages. In this study, high quality oocytes were the grouping of both A and B oocytes as defined earlier. As such, the rest of the oocytes, grade C, were classified as low quality oocytes. Using the aspiration method 193.8 oocytes were collected over the course of the study, of these oocytes

collected only 50.8% were placed into the high quality classification. Whereas with the slicing method, of the 488.6 oocytes collected only 58.7% of those were of high quality. These findings disprove the initial hypothesis provided by the researcher that aspiration would provide more oocytes of quality. These findings were not in accordance with Lekola (2015) who found no significant differences between the quality of bovine oocytes when using aspiration or slicing.

Overall during the collection study, the researcher proved that the slicing method was superior to the aspiration method, in terms of both the quantity and quality for porcine oocytes. Alternatively, Marques *et al.* (2015) found that the aspiration method was the method of choice for later reproductive technologies (p=0.0395). While they found the slicing method to produce a higher quantity of oocytes, their later study showed that there were no differences between oocyte recovery methods until day 7 post IVF. Therefore they showed that maturation rates between the two methods had no significant difference nor did the cleavage rates at day 3 post IVF (Marques *et al.*, 2015).

Further study may be undertaken using different oocyte removal techniques. The two selected were used due to being the more common methods as well as for the ease of the researcher. Other methods such as cutting method could be used, although may be labour intensive with requirements for certain equipment (Hamano & Kuwayama, 1993). The puncture method is another method that may be explored although it appears to have a similar process to the aspiration method (Shirasawa *et al.*, 2013).

5.2 The comparison of maturation media on oocytes' polar body extrusion

Three different *in vitro* maturation media were tested to determine the effect on porcine oocyte maturation rates. With the media being NCSU 37, TCM 199 and follicular fluid (supplemented with FSH and LH). The researcher hypothesised that the media containing follicular fluid would perform better than the chemically based media.

In this experiment, three different maturation media were used due to cost and chemical availability, as there are multitudes of media as well as variations upon those that are well known. The use of porcine follicular fluid was selected with the addition of FSH and LH to try recreate *in vivo* conditions. Porcine follicular fluid inclusion in IVM media has become more popular through the years (Yoshida *et al.*, 1993; Kikuchi & Kawai, 2000). This was then used to determine how much of an effect natural hormones have on the maturation of oocytes when compared to chemical based media. Gil *et al.* (2010) found that the most common media used for porcine IVM was NCSU 37, NCSU 23 and TCM 199; while Pyoos *et al.* (2018) showed that NCSU 37 performed better than NCSU 23 when used in an IVM system. TCM 199 was selected as it was noted by the researcher to be used in many previous studies and was designated as a control for this study. The researcher decided to use the NCSU media but omitted NCSU 23 in favour for NCSU 37 due to the results shown by Pyoos *et al.* (2018); after a similar study was undertaken at the same research site a year prior. The NSCU media contain either taurine, hypotaurine or sorbitol for use in IVM and IVC (Petters *et al.*, 1990; Petters, 1992; Reed *et al.*,

1992; Petters & Wells, 1993; Kikuchi & Kawai, 2000). The NCSU 37 medium that was used in this study was taken from an established protocol available in the laboratory that contained sorbitol.

Maturation of the oocyte was determined by two visual traits seen under the microscope. The first being the expansion of the cumulus oocyte complex (COC) which can be classified into full expansion, partial expansion or partly naked (Alvarez *et al.*, 2009). The ootids that were fully expanded were selected for later work in IVF and ES; an ootid with a fully expanded COC can be seen in Figure 5.2.1. The second indicator of maturation used in this study was the presence of a polar body. The polar body extrusion from an oocyte indicates maturation of the cell following the completion of meiosis I (Pyoos *et al.*, 2018). The ootids were completely denuded and observed under a microscope for the presence of a polar body as seen in Figure 5.2.2 (Alvarez *et al.*, 2009).

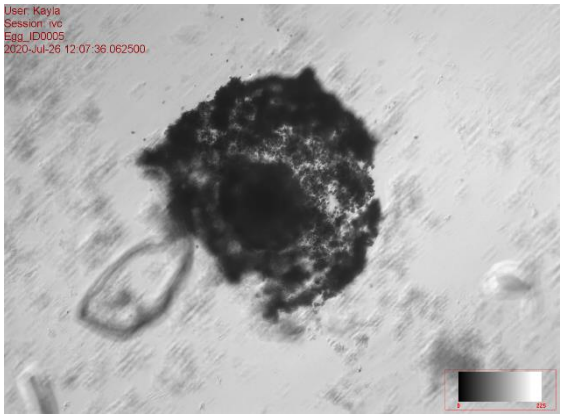

Figure 5.2.1 A cumulus oocyte complex (COC) after 44 hours in the IVM media (4x)

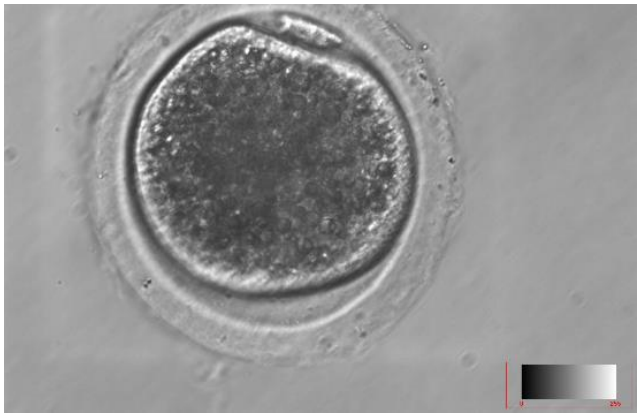

Figure 5.2.2 An ootid with a polar body (40x)

This study found that there were no significant differences (P>0.05) shown between the three treatments selected for IVM (Table 4.2). Upon initial inspection it may seem that there is a large difference with one of the media, NCSU 37. The percentage of polar body extrusion for NCSU 37 was 25.3% of oocytes observed; this value seems low when compared to TCM 199 and mpFF. TCM 199 and mpFF had a polar body extrusion of 84.2% and 73.8% of oocytes observed respectively. Though the figures may seem vastly different when initially read, it has been shown that there are no significant

differences between treatments. This is in contrast to the findings of Pyoos *et al.* (2018) who found that NCSU 37 had significantly higher rates of polar body formation, namely 81.9-85.9%, when compared to the alternative media used in their study. It can be made comparable that the NCSU 37 media used by Pyoos *et al.* (2018) was supplemented with gonadotropins while the other media that performed lesser, approximately 72%, was supplemented with FSH and LH. This can lead the researcher to theorise that the NCSU 37 media used may have performed better in this study if it was enhanced with gonadotropins. Similarly, that FSH and LH may have had an increased effect on cumulus cell expansion when paired with follicular fluid in this study. These findings will need further research in order to determine any validity to the presumptions made by the researcher.

Nevertheless, the potential of IVM media has increased since the early 2000s and this has allowed researchers to collect immature oocytes to place them in the specialized media. This improvement in IVM has allowed for the immature oocytes to have a 20% to 30% chance of progressing to the blastocyst stage (Gil *et al.*, 2010; Appeltant *et al.*, 2016). Therefore the selection of IVM media will have a domino effect on the ability to progress to the blastocyst stage and thereafter, live births. The information obtained through this objective of the study did not allow the researcher to make a conclusion based on the initial hypothesis. Therefore the hypothesis that initially stated that there would be significant differences found between the media treatments cannot be concluded.

It has also been shown that the maturation of oocytes from prepubertal gilts have a lower rate of blastocyst formation when placed in follicular fluid obtained from the same ovaries or other prepubescent gilts (Pawlak *et al.*, 2018). The follicular fluid acquired throughout the study was from different ovaries received on different days, this is due to the low yield of follicular fluid from a singular ovary. This could lead to a variation between the batches of media and discrepancies in the results found (Gadea *et al.*, 2020).

5.3 The comparison of ES on oocytes and frozen-thawed semen on fertilization rates following IVF

The comparison of electrical stimulation on oocytes was carried out to be compared to the effect of frozen-thawed semen on fertilization rates following IVF. Electrical stimulation served as a control for the study of fertilization with semen, therefore it was hypothesised that electrical stimulation would yield higher cleavage rates when compared to IVF. Due to the synchronicity of IVF and IVC, the results of the IVF techniques could not be determined until after IVC had taken place. The oocytes could be viewed immediately after the method was undertaken (Figure 5.3.1 and Figure 5.3.3) as well as immediately before IVC took place (Figure 5.3.2). Though the zygotes could not indicate success prior to IVC taking place.

Polyspermy has always been a major issue for porcine *in vitro* production (Nagai, 1994; Kikuchi & Kawai, 2000; Gil *et al.*, 2010; Romar *et al.*, 2016; Pyoos *et al.*, 2018; De Macedo *et al.*,

2019). This was thought to be due to an issue during the maturation phase of *in vitro* production (IVP) that affects the cortical granule distribution of oocytes (Cran & Cheng, 1986; Nagai, 1994). The rate of polyspermic penetration has been shown to be over 40% in the current porcine IVF systems (Romar *et al.*, 2019; Gadea *et al.*, 2020). However, *in vivo* incidences of polyspermy has been shown to exist when there is an increase in spermatozoa numbers through artificial insemination (Hunter, 1973; Nagai, 1994).

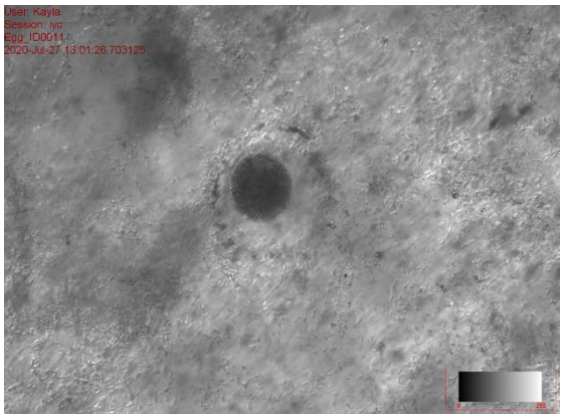

Figure 5.3.1 An ootid surrounded by sperm cells immediately following IVF (4x)

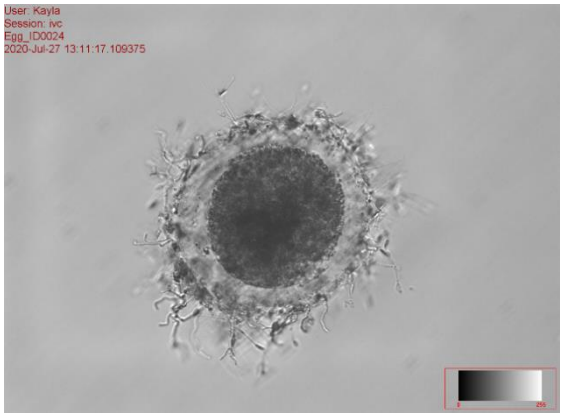

Figure 5.3.2 A zygote surrounded by sperm cells 24 hours post IVF (20x)

Physiologically the normal development of an embryo occurs following fertilization: this comprises of meiotic resumption, cortical granule exocytosis, second polar body extrusion, pronuclear formation and the progression of the cell (Ducibella *et al.*, 2006; Clift & Schuh, 2013; De Macedo *et al.*, 2019). The process of electrical stimulation on matured ootids mimics this physiological progression without the need of spermatozoa. The researcher is then able to determine the ability of the ootids alone to reach the zygote phase and can make assumptions based off of those results if the issue lies with the oocytes, spermatozoa or possibly polyspermy.

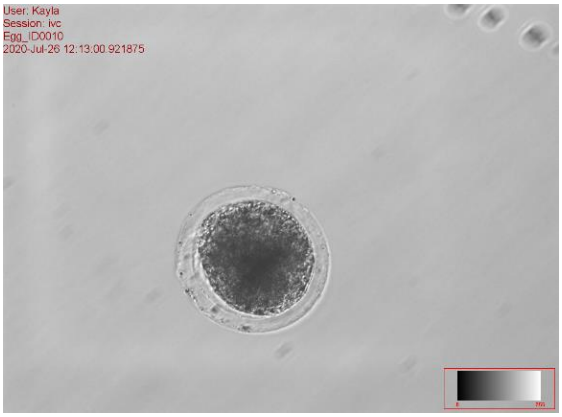

Figure 5.3.3 An ootid immediately post ES (10x)

Following IVF and IVC the zygotes were checked for cleavage, which indicated success, as well as single cells and lysed cells. Single cell zygotes indicated that no growth was observed; this could be due to failure of activation, either through fertilization or ES, or could be due to issues with either of the gametes that prevented further development. Lysed cells were clear indicators of failure due to the death of the cells, the death could be caused by similar issues that inhibit growth but it can only be speculated upon.

This study showed significant differences on cleavage rate of zygotes following fertilization with frozen-thawed spermatozoa and electrical stimulation ($P<0.05$). It was found that the cleavage rate of zygotes for fertilization with frozen-thawed spermatozoa was 48.1% (Figure 4.3.1). While the ES method showed to have a cleavage rate of 56.8% (Figure 4.3.2). The information gained through the study allowed the researcher to conclude that ES had a higher cleavage rate in porcine zygotes which supports the original hypothesis made. These results could lead the researcher to believe that the lower rates of cleavage in IVF when compared to ES could be related to issues with the spermatozoa. One of the issues could be that the spermatozoa may be of lesser quality and may not have the ability to successfully fertilize the ootid. The results found by Lekola (2015) showed a 23% cleavage rate to the 2-3 cell stage when using frozen-thawed spermatozoa. Further, it has been shown recently that fertilization with frozen-thawed spermatozoa can have an 80% farrowing rate *in vivo* (Roca *et al.*, 2011; Didion *et al.*, 2013; Estrada *et al.*, 2014; Gadea *et al.*, 2020).

The aforementioned issues of sperm quality may be summarized by sperm numbers, morphology and motility. Using the CASA-SCA® system, the spermatozoa used in this study had a motility percentage of 49.45%, with a rapid progression of 20.33%. This shows that the motility of spermatozoa used in this study is close to favourable when compared to results of 60-90% found (Quintero-Moreno *et al.*, 2004). With the quality of spermatozoa used in this study being low, with a rapid progression of approximately 20%, this allows the researcher to hypothesize that there is less incidence of fertilization. Therefore the results shown for frozen-thawed fertilization may not be reliable unless replicated with higher quality spermatozoa. Another issue with fertilization with spermatozoa is

polyspermy, this is a well-known issue within porcine reproductive technologies (Agung *et al.*, 2013; Romar *et al.*, 2016); although polyspermic zygotes may advance to the blastocyst stage at the same rate as monospermic zygotes and so are difficult to identify(Han *et al.*, 1999a; b; Gil *et al.*, 2010). Zygotes can be checked for two polar bodies presence 12 hours post fertilization which is indicative of monospermic fertilization (Appeltant *et al.*, 2016). It can also can be made known that the proportion of successful monospermic zygotes would be 45% or less (Romar *et al.*, 2016; Gadea *et al.*, 2020). These findings would allow the researcher to also deduce that the results received for embryo cleavage for the fertilization method may contain numbers of polyspermic embryos but would find it difficult to differentiate between them and healthy embryos. The researcher would then suggest that studies using IVF and ES comparatively are needed to understand more about the issues of polyspermy in the porcine reproductive technology industry.

5.4 The comparison of cell cleavage rates following IVC in two different culture media

Two *in vitro* culture media (cNCSU 37 and cpFF) were selected to determine which would have a higher effect on embryo cleavage rates. cNCSU 37 was selected as it was observed that NCSU 37 and NCSU 23 were previously used but it was decided to only use NCSU 37 to remain consistent within the study. cpFF was selected for similar reasons as well as to determine how much an effect a fluid present in early stages *in vivo* would have on late stage embryo growth. The researcher hypothesized that cNCSU 37 would outperform cpFF. Even though earlier in the study it was thought that a natural medium would be superior to a chemical medium, which was later proven to be incorrect, the opposite is believed to be true for IVC. In this situation the chemically specialized medium would contain the necessary constituents for IVC when compared to a natural medium, such as pFF which would not be present for *in vivo* embryo growth.

In this study significant differences were found between cleavage rate of porcine embryos with the use of the IVC media, cNCSU 37 and cpFF ($P<0.05$). When using cNCSU 37 for IVC the cleavage rate was 66.6% (Figure 4.3.1). In contrast, the results showed that the cleavage rate for embryos when using cpFF was 45.7% (Figure 4.3.2). The IVC of porcine oocytes usually being difficult as the rate of blastocyst formation remains at 30-40% (Gil *et al.*, 2017; Cambra *et al.*, 2020).

As can be seen in Figure 5.4.1 the grouping consists of few healthy zygotes in the cNCSU 37 medium, with the rest beginning to lyse due to cold shock. A healthy embryo in cNCSU 37 can be seen in Figure 5.4.2 which can be confirmed when compared with Figure 2.2.5.2.

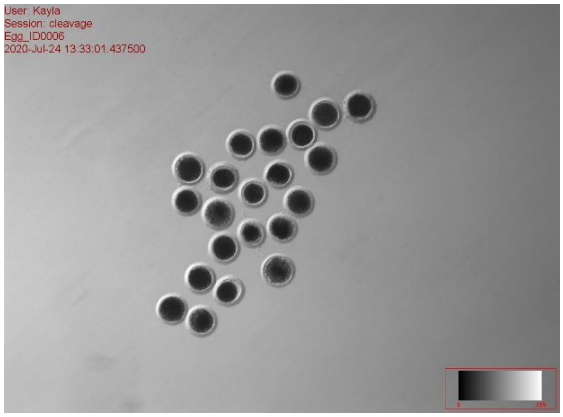

Figure 5.4.1 A grouping of zygotes post IVC in cNCSU 37 (4x)

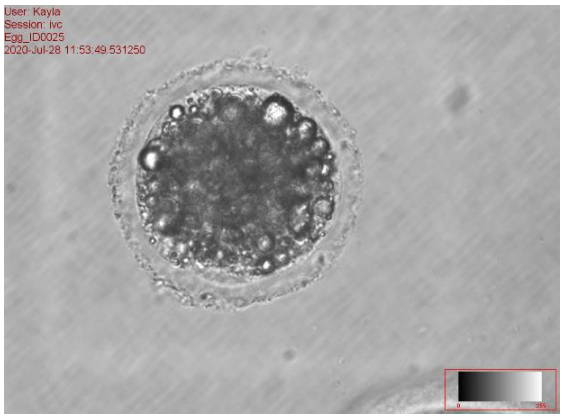

Figure 5.4.2 A healthy embryo following IVC in the cNCSU 37 medium (20x)

This study found that the rate of blastocyst formation was 0%. The researcher noted that the majority of embryo cleavage that did occur was to the two-cell stage (Figure 5.4.3), with few reaching the three cell stage (Figure 5.4.4) and several lysed cells.

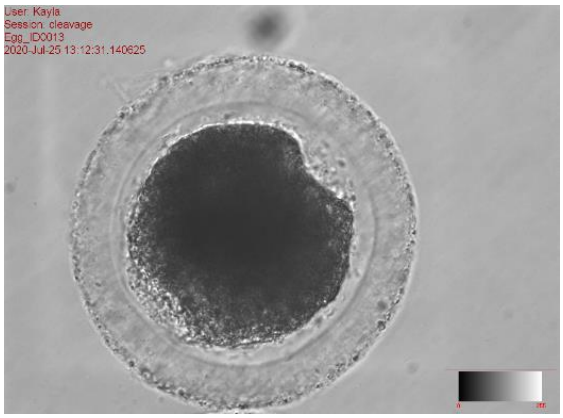

Figure 5.4.3 An embryo advancing to the two cell stage (40x)

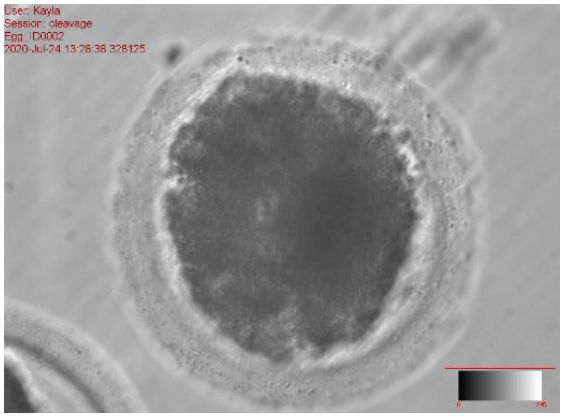

Figure 5.4.4 An embryo advancing to the three cell stage (40x)

As can be seen in Figure 5.4.5 the embryos have a dark appearance which is indicative of cell death. The majority of the replicates where cpFF was used for IVC resulted in this outcome. Therefore, it follows that the presence of porcine follicular fluid, indeed with the modifications, had a negative effect on embryo growth if there is continuous exposure.

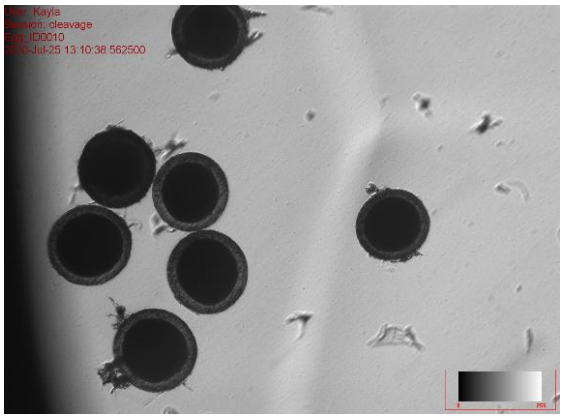

Figure 5.4.5 A grouping of zygotes post IVC in cpFF (10x)

The results of this current study support the hypothesis that cNCSU 37 performs better than cpFF when used in IVC settings. Although, it was found by Duarte *et al.* (2020) that the ability to reach metaphase II following fertilization was increased with the combination of NCSU 37 with pFF (Yoshioka *et al.*, 2008).

Chapter 6: Conclusions

The purpose of this research was to ascertain the more available methods and media used in porcine IVP and to establish which produce the best possible outcomes. This study only included a few protocols and does not encompass the entirety of those methods or media available for porcine IVP.

The goal when recovering oocytes from the ovaries is to obtain the highest possible amount of high-quality oocytes. Due to the porcine ovary being multi-lobulated the potential to collect a large amount of oocytes is high; but due to the ovaries within this study being obtained from prepubertal gilts the quality of the oocytes would be fairly low. It was found through this investigation that the slicing method was significantly better than the aspiration method. The slicing method procured both the highest quantity of oocytes as well as the highest quality oocytes.

The IVM of oocytes is performed to reproduce the *in vivo* conditions that allow the oocyte to mature to a stage where it can be fertilized successfully. The use of mpFF in this study was to fabricate the aforementioned *in vivo* conditions to determine the effect on maturation in IVP. The chemical media, NCSU 37 and TCM 199, were used as comparison to mpFF to determine if there is a superior performance of chemical media in porcine IVP. In this study there were no significant differences found between the three maturation media (NCSU 37, TCM 199 and mpFF) used.

The undertaking of ES and IVF does not guarantee immediate results, therefore the process was combined with IVC to determine success at the blastocyst stage. In this study the zygotes did not reach the blastocyst stage but instead only progressed to the 2-3 cell stage. The researcher found that the ES method had a higher rate of zygote cleavage than IVF. This was as expected that ES should outperform normal spermatozoa fertilization.

The IVC of porcine oocytes is particularly difficult as the success rate of reaching the blastocyst stage is currently shown to be only 30-40%, while in this study there was a 0% blastocyst rate. The use of pFF was used as the researcher theorized that while follicular fluid is important for maturation of oocytes, its continued presence is disadvantageous during culture. The cpFF medium was shown to have lower rates of cleavage when compared to cNCSU 37. Due to cpFF having a large amount of zygotes that were negatively affected by the continued presence of follicular fluid and underwent cell death as seen in Figure 5.4.5.

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Addendum A

A.1 Oocyte washing media

Table A.1.1 The formula for the oocyte washing medium M199

Component	**500ml**
M199 (with Earles salt)	450ml
Foetal bovine serum (FBS)	50ml
Antibiotic Antimycotic	5ml
NaOH (pH adjusted)	3ml (optional)

Table A.1.2 The formula for the oocyte washing medium mDPBS

Component	**500ml**
DPBS	500ml
1% PVA	0.5g
Antibiotic Antimycotic	5ml
Phenol Red	200µl

A.2 *In vitro* maturation media (IVM)

Table A.2.1 The formula for the IVM medium TCM 199

Component	**20ml**
Stock TCM-199	18ml
1% PVA	2g
Na-Pyruvate	0.2g
D-Glucose	0.011g
Cysteine (10mg/ml)	0.2g
EGF (10mg/ml)	20µl

Table A.2.2 The formula for the IVM medium NCSU 37

Component	**g/100ml**
NaCl	0.6356
$NaHCO_3$	0.2106
KCl	0.03563
KH_2PO_4	0.01619
$MgSO_4.7H_2O$	0.02933
$CaCl_2.2H_2O$	0.02499
Glucose	0.01
Glutamine	0.01461
Sorbitol	0.02186
Insulin-Transferrin-Sodium	0.0005
Penicillin-G-Potassium salt	0.0065
Streptomycin	5µl

Table A.2.3 The formula for the IVM medium mpFF

Component	**5ml**
Porcine follicular fluid (pFF)	5ml
FSH	1µl
LH	2µl

A.3 *In vitro* fertilization media (IVF)

Table A.3.1 The formula for the IVF medium BO-IVF stock solution A

Component	**g/100ml**
Deuterium depleted water (DDW)	100ml
NaCl	6.55
KCl	0.3
$MgCl_2.6H_2O$	0.106
$NaH_2PO_4.H_2O$	0.128
Gentamicin	0.5µl
Phenol red	200µl

Table A.3.2 The formula for the IVF medium BO-IVF stock solution B

Component	**g/50ml**
$CaCl_2.H_2O$	7.351

Table A.3.3 The formula for the IVF medium BO-IVF working solution

Component	**g/50ml**
Deuterium depleted water (DDW)	45ml
$NaHCO_3$	0.1552
Caffeine	0.0971
Na-Pyruvate	0.00688
Bovine serum albumin (BSA)	0.2
Stock A	5ml
Stock B	450µl

Table A.3.4 The formula for the electrical stimulation (ES) media

Component	**g/100ml**
Deuterium depleted water (DDW)	100ml
0.3M mannitol	54.6516
Bovine serum albumin (BSA)	0.1
TL-HEPES	5ml

A.4 *In vitro* culture media (IVC)

Table A.4.1 The formula for the IVC medium cNCSU37

Component	**g/100ml**
NCSU 37	100ml
Bovine serum albumin (BSA)	0.4

Table A.4.2 The formula for the IVC medium cpFF

Component	**g/10ml**
mpFF	10ml
Bovine serum albumin (BSA)	0.04

Addendum B

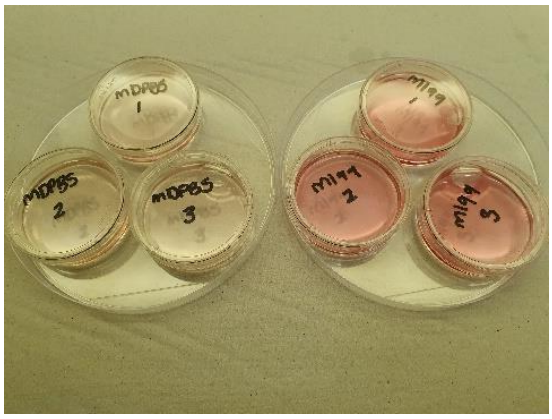


Figure B.1.1 The media used for the oocyte washing procedure

Figure B.1.2 The different IVM media used for oocytes maturation

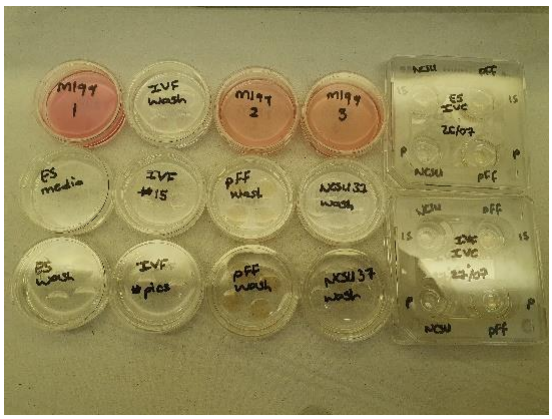


Figure B.1.3 The IVF, ES and IVC media for matured oocytes

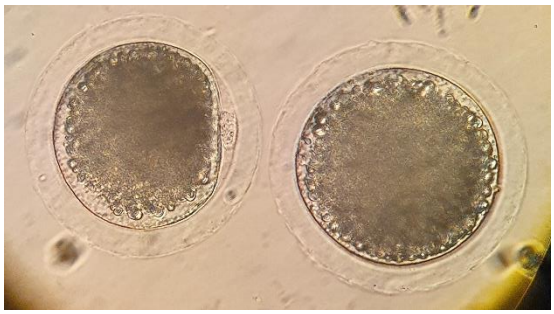


Figure B.1.4 A low quality image of an oocyte with a polar body (left) with an oocyte lacking a polar body (right)

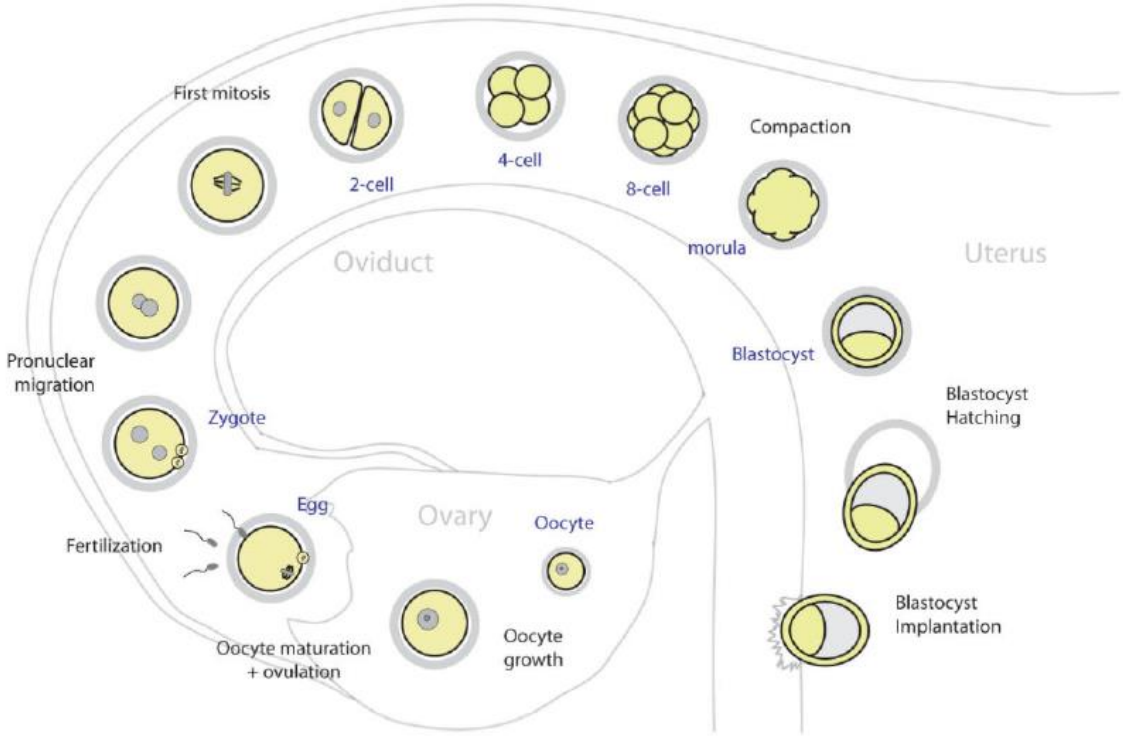


Figure B.1.5 The cell cycle showing the *in vivo* transition from oocyte to blastocyst followed during IVP procedures (Clift & Schuh, 2013)

Maturation	IVF & ES	IVC	Cleavage	Morula	Blastocysts
44 hours	12-15 hours	Day 0 (48 hours)	Day 2 (48 hours)	Day 5 (120 hours)	Day 7 (168 hours)

July 2020

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
28	29 Prepare	30	1 #1 collect and mature	2	3 #1 fertilization & electrical stimulation ES IVC #2 c & m	4 #1 IVF IVC
5 #1 ES cleavage #2 IVF & ES ES IVC	6 #1 IVF cleavage #2 IVF IVC #3 c & m	7 #2 ES cleavage	8 #2 IVF cleavage #3 IVF & ES ES IVC #4 c & m	9 #3 IVF IVC	10 #3 ES cleavage #4 IVF & ES ES IVC #5 c & m	11 #3 IVF cleavage #4 IVF IVC
12 #4 ES cleavage #5 IVF & ES ES IVC	13 #4 IVF cleavage #5 IVF IVC	14 #5 ES cleavage	15 #5 IVF cleavage #6 c & m	16	17 #6 IVF & ES ES IVC	18 #6 IVF IVC
19 #6 ES cleavage	20 #6 IVF cleavage #7 c & m	21	22 #7 IVF & ES ES IVC #8 c & m	23 #7 IVF IVC	24 #7 ES cleavage #8 IVF & ES ES IVC #9 c & m	25 #7 IVF cleavage #8 IVF IVC
26 #8 ES cleavage #9 IVF & ES ES IVC	27 #8 IVF cleavage #9 IVF IVC	28 #9 ES cleavage	29 #9 IVF cleavage	30	31	1

Figure B.1.6 The calendar followed by the researcher throughout the IVF, ES and IVC section of the study

SCA®
SPERM CLASS ANALYZER

Reference: SCAtemp

Date: 2020/11/13
Centre: IVF Laboratory

Code: SCAtemp

Animal: SCAtemp

Ordinary (2020/11/13 04:22:39PM)

Concentration

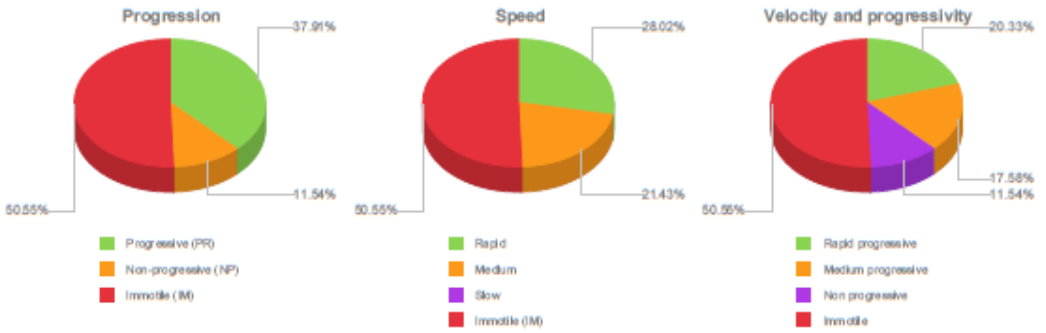
44.50 M/mL	111.25 M/Sample	Volume (mL): 2.50 Dilution 1:0

Progression	Total	Percentage (%)	M/mL	M/Sample
Progressive (PR)	69	37.91	16.87	42.18
Non-progressive (NP)	21	11.54	5.13	12.84
Immotile (IM)	92	50.55	22.49	56.24

	Total	Percentage (%)	M/mL	M/Sample
Motile	90	49.45	22.01	55.01

Speed	Total	Percentage (%)	M/mL	M/Sample
Rapid	51	28.02	12.47	31.17
Medium	39	21.43	9.54	23.84
Slow	0	0.00	0.00	0.00
Immotile (IM)	92	50.55	22.49	56.24

Velocity and progressivity	Total	Percentage (%)	M/mL	M/Sample
Rapid progressive	37	20.33	9.05	22.62
Medium progressive	32	17.58	7.82	19.56
Non progressive	21	11.54	5.13	12.84
Immotile	92	50.55	22.49	56.24



	Average	Immotile (IM)	Slow	Medium	Rapid	Units
Head area	41.32	39.05	44.95	43.43	42.88	μm^2

	Concentration	
Round cells	0.00	M/mL

	Total	ercentage (%
Circular tracks	87	47.80 %

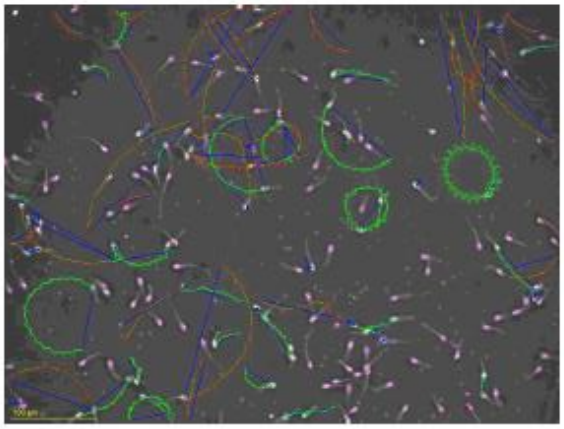
Figure B.1.7 The CASA-SCA® analysis on the semen sample used in the study (1)

Reference: SCAtemp | Code: SCAtemp | Animal: SCAtemp | Date: 2020/11/13
Centre: IVF Laboratory

Avg. values of speed	Motile	Non progressive	Medium progres	Rapid progressiv	Units
Curve speed - VCL	146.74	45.49	141.38	208.85	µm/s
Avg. value - VAP	103.22	22.52	95.28	155.89	µm/s
Linear speed - VSL	78.55	10.30	54.24	138.32	µm/s
Straightness index - STR	70.44	42.33	68.27	88.28	%
Linearity index - LIN	51.60	22.60	53.02	66.83	%
Oscillation index - WOB	68.73	49.28	73.90	75.31	%

Avg. values of other parameters	Motile	Medium progressive	Rapid progressive	Units
Amplitude lateral head - ALH	2.85	2.88	3.46	µm
Beat frequency - BCF	22.81	23.56	29.13	Hz

	Total	Percentage (%	Percentage (%	M/mL	M/Sample
Hyperactive	11	12.22	6.04	2.69	6.72
Mucous penetration	38	42.22	20.88	9.29	23.23



Technician: Surname2, Name2
Comments:

Figure B.1.8 The CASA-SCA® analysis on the semen sample used in the study (2)