IMPROVEMENT OF PORCINE GENETIC RESOURCE PRESERVATION BY IMPROVING *IN VITRO* PRODUCTION, VITRIFICATION AND TRANSFER OF EMBRYOS

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ABSTRACT

One of the main causes of poor *in vitro* embryo development (IVEP) efficiency is incomplete cytoplasmic maturation of pig oocytes. In contrast to nuclear maturation, there are no efficient markers to detect the cytoplasmic maturation. The overall objective of this dissertation was to investigate the effect of sorting oocytes using mitochondrial (MT) and cortical granules (CGs) distribution as cytoplasmic maturation markers of selected mature oocytes on improving embryonic development, polyspermy, embryo vitrification and transfer them to recipients. We hypothesized that embryos derived from sorted oocytes would be better than non-sorted oocytes in embryo development, quality, and viability after vitrification. For this purpose, we used MitoTracker green (MTG) and Peanut agglutinin (PNA) as fluorescent dyes to sort the oocytes based on MT and CGs distribution, respectively.

In chapter 2, we examined the viability rate of oocytes after using of some fluorescent dyes. We used Propidium Iodide (PI) to check the viability rates of oocytes after staining with MTG, PNA and Rhodamine-123 (RH-123). Also, we used Fluorescein diacetate (FDA) to examine MitoTracker orange (MTO) and wheat germ agglutinin (WGA) for oocyte viability. We evaluated the viability rate then the MT and CGs distribution for oocytes derived from pre-pubertal gilts and adult sow pigs using both confocal and wide-field microscopes to find if the wide-field microscope is an adequate replacement to confocal microscope. We found that the concentrations of 200 nM of MTG and 625 nM of PNA were optimal to use in the next series of experiments with maximum visibility and viability. The viability rates for these concentrations were 94.7% ± 5.10 (MTG 200 nM) and 90.9% ± 0.83 (PNA 625 nM). Also, we found that the distribution of MT and CGs can be recognized using wide-field microscope in the same efficiency of confocal microscope. There was strong agreement between the wide-field and confocal microscopy (>96%) using MTG and PNA for pre-pubertal oocytes. In addition, there was no significant differences in MT and CG distribution using oocytes derived from pre-pubertal or sow pigs using wide-field microscope.

In chapter 3, we developed a technique to segregate mature oocytes from harvested oocytes using MTG and PNA staining using wide-field microscope. We investigated the *in vitro* development of embryos, quality, and polyspermy to embryos derived from sorted and non-sorted oocytes. There were no significant differences between the matured sorted (diffused MT

or peripheral CGs) and non-sorted oocytes in cleavage, morula, and blastocyst stages as well as the polyspermy. On the other hand, the quality of blastocysts was better morphologically in embryos derived from sorted oocytes than non-sorted oocytes. In addition, the polyspermy was significantly ($P \leq 0.05$) less in matured oocytes and non-sorted oocytes groups than immature patterns or non-inseminated groups.

In chapter 4, we evaluated many maturation media and their effects on MT and CGs distribution, IVEP, and polyspermy. The media evaluated in this chapter were Tissue Culture Medium-199 (TCM-199), modified Whitten's Medium (mWM), North Carolina State University 23 (NCSU23), North Carolina State University 37 with glucose (NCSU37G), and North Carolina State University 37 with pyruvate and lactate (NCSU37PL). There were no significant differences among the media used in this chapter in MT and CGs distribution, cleavage, morula, and blastocyst rates. On the other hand, the NCSU37PL had less ($P \leq 0.05$) polyspermy than other media in both matured sorted oocytes and non-sorted oocytes groups.

In chapter 5, we evaluated the effect of MT-sorted oocytes and CG-sorted oocytes (mature pattern) on the viability, quality, and development of embryos after the vitrification and warming procedures. Also, we tried to examine the embryo transfer (ET) process for both vitrified and non-vitrified (fresh) morulae. We found that the vitrification and warming procedures had a negative affect ($P \leq 0.05$) on embryos viability, quality, and development for both sorted and non-sorted groups. However, there was no difference between sorted and non-sorted oocytes according to the previous parameters. In addition, there was no difference between the vitrified embryos and fresh embryos on the pregnancy after ET (no pregnancy for both groups), but one of the gilts in vitrified group had numerous corpora lutea when they checked by necropsy.

In conclusion, results from the present dissertation support the hypothesis partially. The sorting of oocytes can be done using wide-field microscopy with fluorescent dyes such as MTG and PNA, and it is a useful technique to obtain high quality embryos after IVEP with less polyspermy. There is no significant effect of maturation media on IVEP outcome. We suggest additional experiments to improve and develop the vitrification and warming procedures as well as ET with a larger number of animals.

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DEDICATION

I dedicate this work to a special person in my life …. my wife. I have no enough words to describe my feelings. Without your support, I've never got the degree.

I dedicate this work also to my kids Ali, Mohammed, Maryam, and Sarah …. Without you and your love, I never been patient to finish my work.

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μm:	Micrometer
μM:	Micromoles per liter
BSA:	Bovine serum albumin
cAMP:	Cyclic adenosine mono phosphate
CG(s):	Cortical granule(s)
COC(s):	Cumulus oocyte complex(es)
EGF:	Epidermal growth factor
ET:	Embryo transfer
FSH:	Follicle stimulating hormone
IVC:	*In vitro* culture
IVF:	*In vitro* fertilization
IVM:	*In vitro* maturation
IVEP:	*In vitro* embryo production
LH:	Luteinizing hormone
mM:	Millimole
MT:	Mitochondria
mTBM:	Modified Tris-buffered medium
MTG:	MitoTracker green
MTO:	MitoTracker orange
mWM:	Modified Whitten's medium
NCSU23:	North Carolina State University 23
NCSU37G:	North Carolina State University 37 with glucose
NCSU37PL:	North Carolina State University 37 with pyruvate and lactate
nM:	Nanomole
PBS:	Phosphate buffered saline
pff:	Porcine follicle fluid
PI:	Propidium iodide
PNA:	Peanut agglutinin
PVA:	Polyvinyl alcohol

RH-123:	Rhodamine-123
SD:	Standard deviation
TCM-199:	Tissue culture medium-199
WGA:	Wheat germ agglutinin
ZP:	Zona pellucida

CHAPTER 1: GENERAL INTRODUCTION, HYPOTHESIS, AND OBJECTIVES

In 2021, there were many endangered porcine breeds (36-115 female stocks) or critical breeds (1-35 female stocks) in North America including Gloucestershire Old Spots, and Guinea Hog as endangered breeds and Large Black, Choctaw, Meishan, Mulefoot, Ossabaw Island, Berkshire, Hampshire, Lacombe, Red Wattle and Tamworth as critical breeds (The Livestock Conservancy, 2021). In Canada, the number of pig farms decreased from 63,602 in 1976 to 7,575 pig farms (Statistics Canada, 2021). Many techniques such as artificial insemination, intra-cytoplasmic sperm injection (ICSI), *in vitro* embryo production (IVEP), vitrification of embryos, and embryo transfer (ET) through embryo banking can improve genetic resources in the porcine industry and preserve breeds. In addition to enhancing genetic resources, these techniques can improve biosecurity and prevent the spread of diseases, especially during international transport, by selecting pathogen-free embryos. The production of embryos *in vitro* consists of three steps: *in vitro* maturation of oocytes (IVM), *in vitro* fertilization (IVF) of matured oocytes, and *in vitro* culture (IVC) of presumptive zygotes. While IVEP techniques reached an accepted level in bovine in the 1980s (GRUPEN, 2014), the success of IVEP in porcine is still low (MARTINEZ et al., 2015a). In porcine, the IVEP procedures still face numerous issues that are unsolved today including: inadequate cytoplasmic maturation, small percentages of embryo development, and fertilization irregularities such as polyspermy. Each step in the IVEP procedure has problems leading to a decrease of IVEP efficiency. Pre-selecting embryos before transfer will control the quality of the piglets. Unfortunately, the previous techniques are not optimized and produced lower percentages of embryos in pigs compared to bovine so they must be enhanced for usage by the swine industry. The quality of oocytes affects the number of embryos produced and their quality. Better quality oocytes produces higher quality embryos (DUSZEWSKA et al., 2012). Using cytoplasmic staining methods could be a way to choose high competence oocytes. The IVEP can be improved if the maturation step is also improved. High quality oocytes will culminate with high quality embryos that can survive vitrification and ET procedures. The research described in this thesis will address deficits in knowledge of cytoplasmic maturation markers to assist in improving IVEP.

1.1. Oocyte Development and Maturation *In Vivo* and *In Vitro*

The process of oocyte development or maturation (oogenesis) is the transformation of germ cells into different stages of oocytes as primary, secondary, tertiary, and lastly the ovum (MONTI and REDI, 2013). This process begins in the embryonic stage when primordial germ cells start migrating through the hindgut and dorsal mesentery to reach the gonads, and undergo a significant mitosis increasing in their number (OKTEM and OKTAY, 2009). When primordial germ cells reach the developing embryonic female genital ridges, they continue to spread (proliferate) as oogonia and then enter meiosis (HUMMITZSCH et al., 2013). In porcine, meiotic division begins about 40 days of embryonic gestation, and all oogonia are in prophase (the germinal vesicle stage) of the first meiotic division (MI) and arrest at around 35 days after birth (HUNTER, 2000). Oogonia undergo the diplotene stage of meiosis I and generate primary oocytes that remain arrested until they are released during ovulation (GULIMIHERANMU et al., 2021). The primary oocytes will be surrounded by pre-granulosa cells (flattened cells in single layer) to generate what is called primordial follicles (WANG et al., 2017, FORTUNE, 2003). When the primordial follicles increase in size and the flattened pre-granulosa cells surrounding the primary oocytes transform to cuboidal, the follicles form into primary follicles (SKINNER, 2005, MCGEE and RAJ, 2015). These oocytes are still inactive inside the follicle for long time until the event of puberty stimulates meiosis resumption (PICTON, 2001).

The primary oocytes will change significantly, by increasing the number of mitochondria (MT) to support these oocytes with energy required in growth, the movement of Golgi apparatus to the peripheral area, and significant increases in the size and formation of the zona pellucida (ZP) between the granulosa cells and the oolemma (PAULINI et al., 2014). These changes to the primary oocyte will transform the primary follicles into the early secondary follicles that contain two layers of cuboidal granulosa cells. The oocytes will continue growing to have five layers of granulosa cells with the accumulation of fluids inside the granulosa cells resulting in an early antral follicular stage.

The development from primordial to the early antral follicle can take many months. This development is gonadotropin independent. The development from primordial to secondary (preantral) stage, through the primary follicles, is gonadotropin independent (PICTON , 2001,

GEORGES et al., 2014). Folliculogenesis can be divided into three major stages: early follicle growth, transformation from preantral (secondary) to antral phase (tertiary), and growth/maturation towards ovulation. The last two stages are dependent on gonadotropins (RECCHIA et al., 2021). On the other hand, a small initial level of steroid hormones could be required for follicle development, but they have inhibitory effects only at high concentrations (DUTTA et al., 2014). It is believed that the primordial follicle activation and proliferation is estrogen independent (BRITT et al., 2004). It is unclear until now if estrogen regulates early follicular growth and the stimulating action of FSH on follicle growth is regulated by estrogen (CHI and CAO, 2021). At the beginning of puberty, the female starts to produce and release steroid hormones that have responsibility for reproduction as well as gonadotropins which include luteinizing hormone (LH) and follicle stimulating hormone (FSH). There are also physiological changes that will happen in a relationship with the estrous cycle while starting puberty. In this stage, as an opposite case with prepubertal period, the development of oocytes is hormone dependent, and is controlled essentially by interaction between estrogen and progesterone (the steroid hormones) that are produced inside the ovaries in addition to the gonadotropins that are produced and released from the anterior side of the pituitary gland (COLVIN and ABDULLATIF, 2013). These gonadotropins are released and controlled by a hypothalamic hormone called gonadotropin releasing hormone (GnRH) that influences the gonadotroph cells in anterior pituitary to make and release the LH and the FSH hormones. These hormones (LH and FSH) regulate and trigger the growth of follicles (LOMNICZI et al., 2013).

When the follicles mature, the theca interna cells within the follicles will start to synthesize androgens. Androgens are used to make estradiol through the P450 aromatase enzyme in the granulosa cells of the ovarian follicles (HATZIRODOS et al., 2014, SLOMCZYNSKA and TABAROWSKI, 2001). The estradiol levels will be stimulated by FSH directly. For the larger follicles, they produce more estradiol which can stimulate more receptors of FSH on follicle membranes (KWINTKIEWICZ et al., 2010). This stimulating mechanism leads to increased FSH circulation to support follicle growth. On the other hand, inhibin, the inhibitory hormone that inhibits FSH production, is released from the antral follicles which limit the release of FSH. For competent follicles only, there are sufficient numbers of FSH receptors to remain viable (PICTON, 2001). This mechanism means a good selection process to choose competent follicles. There are many significant modifications that happen in the follicles within this

selection mechanism. Firstly, the antral fluid increases into a huge amount to fill the antrum through the late antral phase and stimulated by the FSH. Secondly, the granulosa cells (GCs) start to separate into two groups. The inner group that surrounds the oocyte will become cumulus cells, while the outer group will be mural granulosa cells ((GOSDEN, 2013, DUNLOP and ANDERSON, 2014, GEORGES et al., 2014); Figure 1.1). The cumulus cells remain connected with the oocyte, which forms the cumulus oocyte complex (COC). Proliferating GCs progressively form several layers around the oocyte in the secondary to late preantral follicles (GERSHON and DEKEL, 2020).

From this stage, a layer of theca cells surrounds the follicle, and the follicles start to produce estrogens. Theca cells produce androgens, which are converted into estrogens in granulosa cells. Whereas follicular growth to the secondary/preantral stage is independent on gonadotrophins, progression beyond this stage strictly depends on FSH stimulation (ALLAN et al., 2006). At this stage, selection occurs between growing follicles, and only one or a limited number of follicles continue growing to the preovulatory stage while others undergo atresia. Two layers of theca (theca interna and externa) can be distinguished around selected follicles. After ovulation, which is triggered by a peak of LH, theca cells and mural granulosa cells luteinize to produce progesterone (GEORGES et al., 2014, HSUEH et al., 2015). This mechanism is suggested for the mono-ovulatory species as well as for poly-ovulatory species, but the mechanism itself is still unclear (MIGONE et al., 2016).

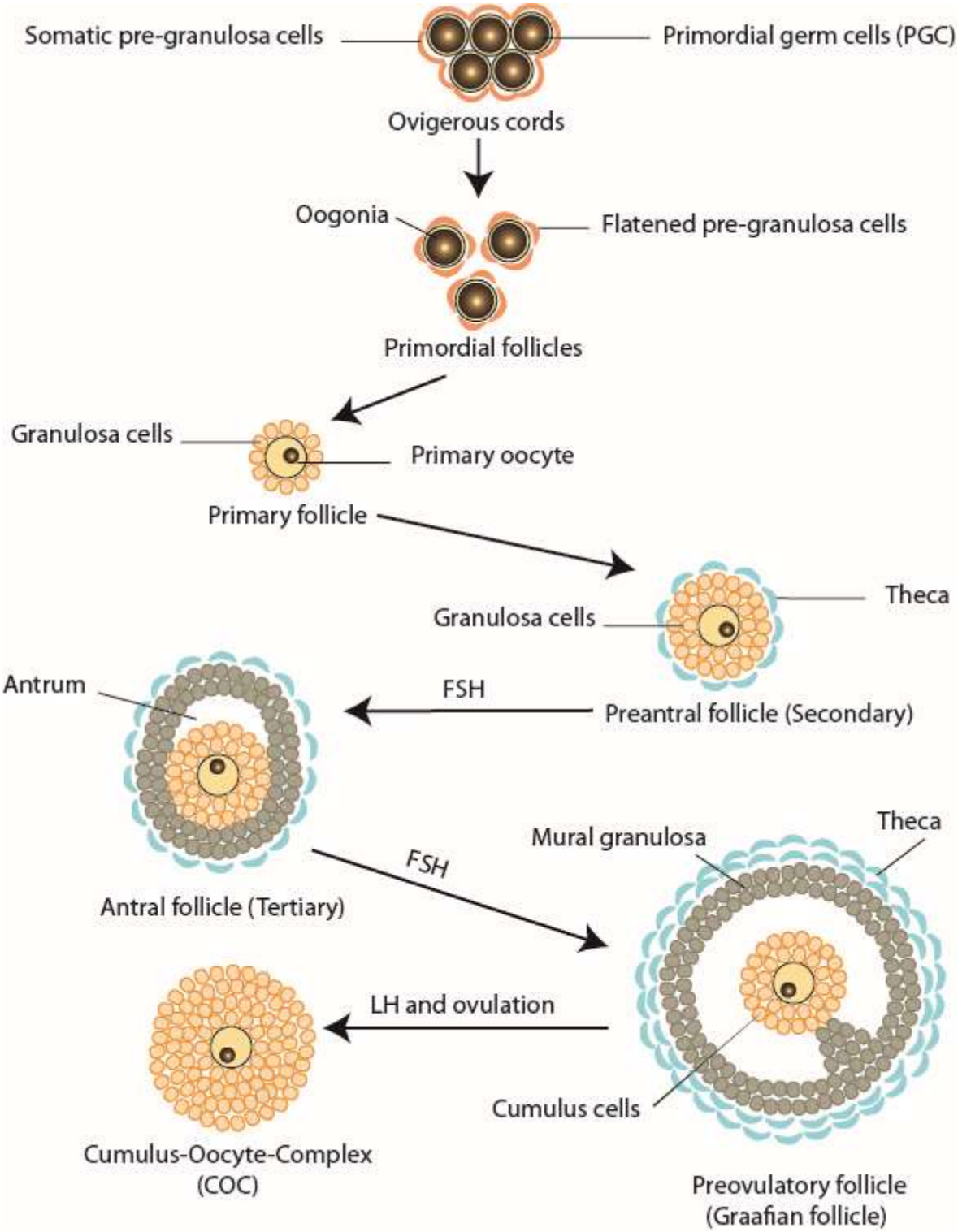

Figure 1.1: Outline of the main steps of folliculogenesis which starts with primordial germ cells (PGC) throughout ovulation until the cumulus-oocyte-complex (COC). Abbreviations: FSH, follicles stimulating hormone; and LH, luteinizing hormone.

As mentioned before, the increase of antral follicle numbers will increase estradiol levels, and then increase FSH level. This increase in estradiol levels has a positive effect on GnRH to stimulate LH to transform the mature follicles and its theca interna into a corpus luteum after ovulation (MOENTER et al., 2020). The LH function in this stage is to help the oocyte mature, and to prepare the oocyte for ovulation. This maturation occurs for both the nucleus and cytoplasm.

The female reproductive system gives the perfect environment and element of biochemical signals that leads the changes of the oocyte through maturation, fertilization, and development of embryos. Mimicking the *in vivo* environment is challenging in *in vitro* conditions. Several factors or problems can affect IVM, and they may limit its success. First, the quality of the oocyte itself has effects on IVM. The oocyte must have some characteristics to be mature and to be ready for fertilization. For example, follicles size affects IVM significantly, so follicle sizes larger than 8 mm were better than medium size (3-7 mm) after 18 hours of IVM (KWAK et al., 2014). Moreover, the cytoplasmic and nuclear maturation must occur simultaneously, although nuclear maturation tends to be faster and usually precedes cytoplasmic maturation (GRUPEN, 2014). Unfortunately, cytoplasmic maturation is a significant problem in IVM because it is not easy to determine this maturation directly, unlike nuclear maturation. Another factor that can effect IVM in porcine is the age of the animal that is the source of oocytes. NAGAI (2001) found that oocytes collected from ovaries of adult animals were more developed compared to oocytes collected from younger animals. Beside oocyte and animal factors, another major problem in porcine IVM is the culture medium in which oocytes are placed for maturation due to insufficient maturation media for IVEP (NAGAI et al., 2006). Finally, using oocytes with heterogeneous cytoplasm had more blastocysts obtained than oocytes with homogeneous cytoplasm (NAGAI, 2001). It is understood that removal of the oocyte from antral follicles results in the unconstrained resumption of meiosis as independent of LH secretion.

It is likely that decreasing the adenosine cyclic monophosphate (cAMP) levels in oocyte *in vitro* is the main factor of this quick maturation through maturation promoting factor (MPF) destabilization, which decreased in *in vitro* matured oocytes (SAHU et al., 2018). Along these lines, control of intra-oocyte cAMP and its wide signal system is critical to recreate *in vivo* conditions, and at last, the inability to do as such has brought about the present imperfect IVM of

oocytes (RICHANI et al., 2019). It is necessary to know how *in vitro* conditions present in abnormal development. Surprisingly, that cAMP inhibitors can be secreted from the oocyte itself, not from cumulus or granulosa cells only (SELA-ABRAMOVICH et al., 2006). Loss of the G-protein coupled receptor 3 and 12 (GPR3) and (GPR12) will decrease cAMP produced endogenously, and then loss of natriuretic peptide precursor type C (NPPC) will decrease cyclic guanosine monophosphate (cGMP) and produce oocyte-specific phosphodiesterase 3A (PDE3A) to active cAMP store degradation (KAWAMURA et al., 2011). Besides the loss of NPPC, the cGMP decreasing *in vitro* might be because of complete loss of functions in gap junctions before several hours of culturing.

Gap junction loss seems to delay *in vitro* induction of cAMP, and it is certain that this molecule is important for suitable maturation (THOMAS et al., 2004). The cAMP as a transmitter of LH action can induce epidermal growth factor (EGF) through the activation of the mitogen-activated protein kinase (MAPK) that may work as an intrafollicular transmitter to enhance the COC expansion and oocyte maturation (FAN et al., 2009). The reason for the deficiency of cumulus cell expansion in matured oocytes *in vitro* could be because of the irregularities in MAPK pathways and this could cause IVM oocyte abnormalities (SU et al., 2003). As a result of this sustained arrest *in vitro* is that the IVM oocytes are collected from follicles at different stages of maturation, from early antral to atretic, and in this way the development of the gathered oocyte groups is very heterogeneous.

1.1.1. Nuclear and Cytoplasmic Maturation

Nuclear maturation starts when germinal vesicles (GV) of primary oocytes, which are in diplotene stage of prophase I, restart meiosis and then go through germinal vesicle breakdown (GVB) or (GVBD) (ZHANG and XIA, 2012). The ability to resume and complete meiosis is known as meiotic competence. GVB is quickly followed by arrangement of spindles in metaphase I, condensation of chromosomes, segregation of homologous chromosomes and expulsion of the first polar body, in this way finishing meiosis I. The oocyte in this stage will start meiosis II and remains as a secondary oocyte in metaphase II (MII) until fertilization. In this stage, the pre-ovulatory follicles will be ruptured as a response to LH that helps to release the oocyte into the oviduct. When the sperm connect with the oocyte in the ampulla at fertilization

time, meiosis and the rearrangement of the diploid zygote will be finished (KANE, 2003). The remaining cells of the ruptured follicle, such as mural granulosa and theca interna, will transform to produce progesterone producing cells in corpus luteum (HAVELOCK et al., 2004). Although increasing LH levels stimulates the maturation *in vivo*, nuclear maturation happens after the removing of antral cells from the oocytes *in vitro* (SIRARD et al., 2006).

Cytoplasmic maturation means that molecular modifications can happen in the ooplasm of an oocyte leading to competence of the oocyte and its ability to be fertilized. These modifications include redistribution of mitochondria, ribosomes, endoplasmic reticulum, and Golgi apparatus. This redistribution supports the store of maternal messenger ribonucleic acid (mRNA), increasing the synthesis of proteins and other modifications to complete cytoplasmic maturation (WATSON, 2007). The real store of mRNA is serious, as this store of mRNA should support the embryo until the maternal to zygotic transition stage (SHEN-ORR et al., 2010). Despite the fact that mRNA development happens all through folliculogenesis, it stops once nuclear maturation resumes and the oocyte advances to GVB (VITALE et al., 2007). Since the oocyte can continue meiosis and completion nuclear maturation preceding the finish of cytoplasmic maturation, it is important that this store of maternal mRNA happens before the end of oocyte mRNA synthesis brought about by the reinitiating of meiosis (SHA et al., 2019). Finishing cytoplasmic maturation is necessary to complete full oocyte maturation. The main cause of the low rate of embryos from IVM of oocytes is incomplete cytoplasmic maturation in parallel with nuclear maturation, since the nuclear maturation occurs before cytoplasmic maturation (BANWELL and THOMPSON, 2008).

1.1.2. Meiotic Arrest and Resumption

There are many pathways to explain why the follicles sustain meiotic arrest before the increase in LH levels. High concentrations of cAMP inside the COC can inhibit the signals that maintain meiotic arrest while folliculogenesis proceeds (MEHLMANN, 2005). In fact, these signals can also stop meiotic resumption after oocyte development to MII. The meiotic competence is achieved by the oocyte inside the early antral follicle; however, cAMP signals maintain arrest all through process to the maturation of the antral stage (RICHANI et al., 2019). The inhibition can be cancelled by LH secretion before ovulation and development of the corpus

luteum. There are many mechanisms to describe the high concentration of cAMP as a second messenger inside follicles. cAMP can be produced by cumulus cells to diffuse into the oocyte via gap junction channels (LODDE et al., 2013). Although high concentrations of cAMP exist between the oocyte and the cumulus cells, it is thought that this diffusion is not a main source in the oocyte. It is possible to block intra-oocyte cAMP biosynthesis by removal of both GPR and PDE simultaneously. There are unique GPR3 and GPR12 orthologs in mice and rats, respectively; PDE3A is the most common PDE across species (HINCKLEY et al., 2005). GPR activity is important and adequate to keep meiotic arrest (MEHLMANN et al., 2004).

The NPPC also has a specific expression in the mural granulosa cells and helps in meiotic arrest. The NPPC binds to the cognate guanylyl cyclase receptors (NUR2 and NPR2) which are expressed in cumulus cells to stimulate estradiol and FSH, and then produce cGMP in the cumulus cells (ZHANG et al., 2011). Increasing cGMP diffuses into the oocyte through gap junctions and acts as a strong inhibitor of PDE3A and stimulates the supported store of cAMP produced endogenously (HANNA et al., 2012). The cGMP differs from cAMP in that cGMP is not produced inside the oocyte, so the connection between the cumulus cells and the oocyte is required to produce cGMP unlike cAMP (BU et al., 2004). The NPPC is just ready to anticipate gonadotropin-independent nuclear maturation in COC, but not the denuded oocytes. This impact is probably that the oocytes themselves require NUR2/NPR2 receptors and then cannot have a response to bind with NPPC to increase the cAMP. Despite the fact that the oocytes had continued meiosis in formed follicles, the cumulus cells and granulosa cells were still immature and firmly packed, showing no indications of gonadotropin stimulation (ZHANG et al., 2011). Subsequently, NPPC and its related receptors assume a basic part in the inhibition of oocyte maturation. The high concentration of cAMP in oocyte affects through cAMP-dependent protein kinase A (PKA) to keep MPF inactive. The MPF is a heterodimer kinase responsible to enable the transfer from the growth 2 phase (Gap 2; G2) to metaphase to begin the GVB stage. It is believed that PKA controls MPF activity by starting the activation of MPF through the phosphorylation of mitosis inhibitor protein kinase and myelin transcription factor 1 (Wee1/Myt1) (ZHANG et al., 2009) (Figure 1.2). The Wee1/Myt1 are responsible to dephosphorylate the cyclin-dependent kinase 1 and cyclin B complex (CDK1/CyB) and then deactivate the MPF so the oocyte will be arrested at the GV stage

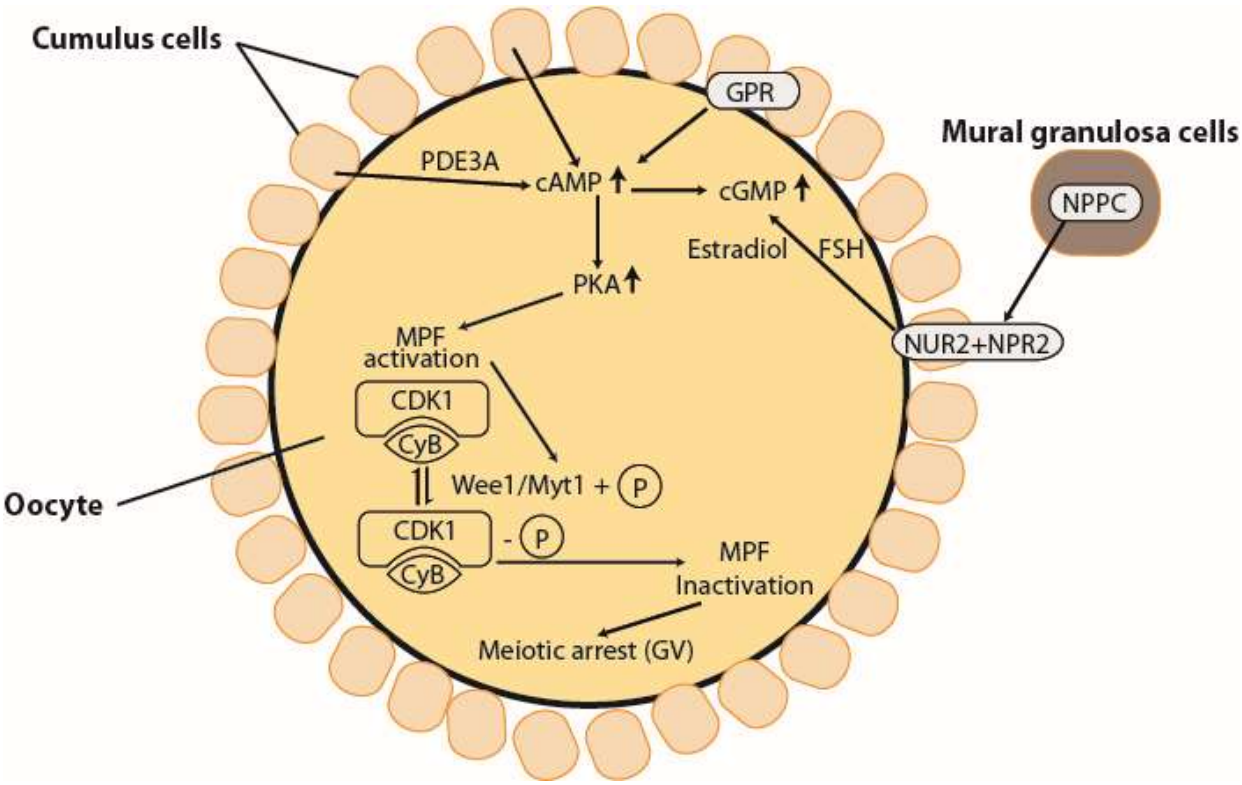

Figure 1.2: Regulation of oocyte meiotic arrest in mammals. Abbreviations: NPPC, natriuretic peptide precursor type C; NUR2 and NPR2, cognate guanylyl cyclase receptors; GPR, G-protein-coupled receptor; FSH, follicular stimulating hormone; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate; PDE3A, phosphodiesterase 3A; PKA, protein kinase A; CDK1, cyclin-dependent kinase 1; CyB, cyclin B; MPF, maturation promoting factor; Wee1, mitosis inhibitor protein kinase 1; Myt1, myelin transcription factor 1; and GV, germinal vesicle stage. Circled P with + indicates to phosphorylation, and circled P with – indicates to dephosphorylation.

Gap junctions are responsible for propagating the paracrine and autocrine signals connected with the increasing LH levels that stop the nuclear arrest and affect final oocyte maturation. The LH hormone directly binds LH receptors (LHR) in the theca and mural granulosa cells to prepare for ovulation. In spite of the significant impact of LH on the oocyte and cumulus cells, these cells do not express LHR themselves. Accordingly, all LH-intervened consequences for these cells should be completed by implication through paracrine, juxtacrine or autocrine signals. EGF is released into the antral fluid by proteolytic cleavage from the granulosa cells upon activation of the LHR (PARK et al., 2004). There are three types of EGF which are amphiregulin (AREG), betacellulin (BTC), and epiregulin (EREG), and rapid mRNA expression for these three EGF types happens *in vivo* after LH stimulation (HSIEH et al., 2007). This expression does not stay more than 3 hours, 6 hours, and 12 hours for AREG, BTC, and EREG respectively (PARK et al., 2004). In fact, the EREG and AREG can stimulate LH secretion. As a result of the binding of gonadotrophins to their receptors in the cumulus granulosa cells, cAMP generation is increased. By activating the PKA pathway in the cumulus cells, the cAMP-responsive element-binding protein (CREB) is regulated (WANG et al., 2016). This leads to the formation of transmembrane precursors of EGF-like factors, which are subsequently converted into mature peptides by the Protein kinase C (PKC) pathway, potentially (CHEN et al., 2014). Via phosphoinositide 3-kinase and protein kinase B (PI3K/PKB) as well as downstream steroidogenesis, EGF-like factors activate MAPK by binding to the EGFR binding site in cumulus cells (KRAUS et al., 2020). Due to phosphorylation of Connexin (CX43) and the activation of PDE3A (ZHANG et al., 2015), activated MAPK and PI3K/PKB reduce cAMP levels in the oocyte so meiosis can resume (Figure 1.3). If Nitric Oxide (NO) and/or NPPC are stimulated by FSH, they may help prevent early oocyte maturation, while LH stimulation decreases this second messenger and contributes to ovulation.

Another result was found that the absence of EGF can prevent the maturation of oocytes, but this effect of EGF is on cumulus cells not inside the oocyte. This result can be caused by MAPK that will activate the extracellular signal-regulated kinase (ERK) through the phosphorylation of serine and threonine protein kinases (OHM et al., 2019). This phosphorylation is very important to start the meiotic resumption of oocytes, and then leads to the GVB stage (LIANG et al., 2007). On the other hand, the MAPK could be activated by EGF binding inside the oocyte itself to induce GVB maturation, but not start meiosis (NI et al., 2015).

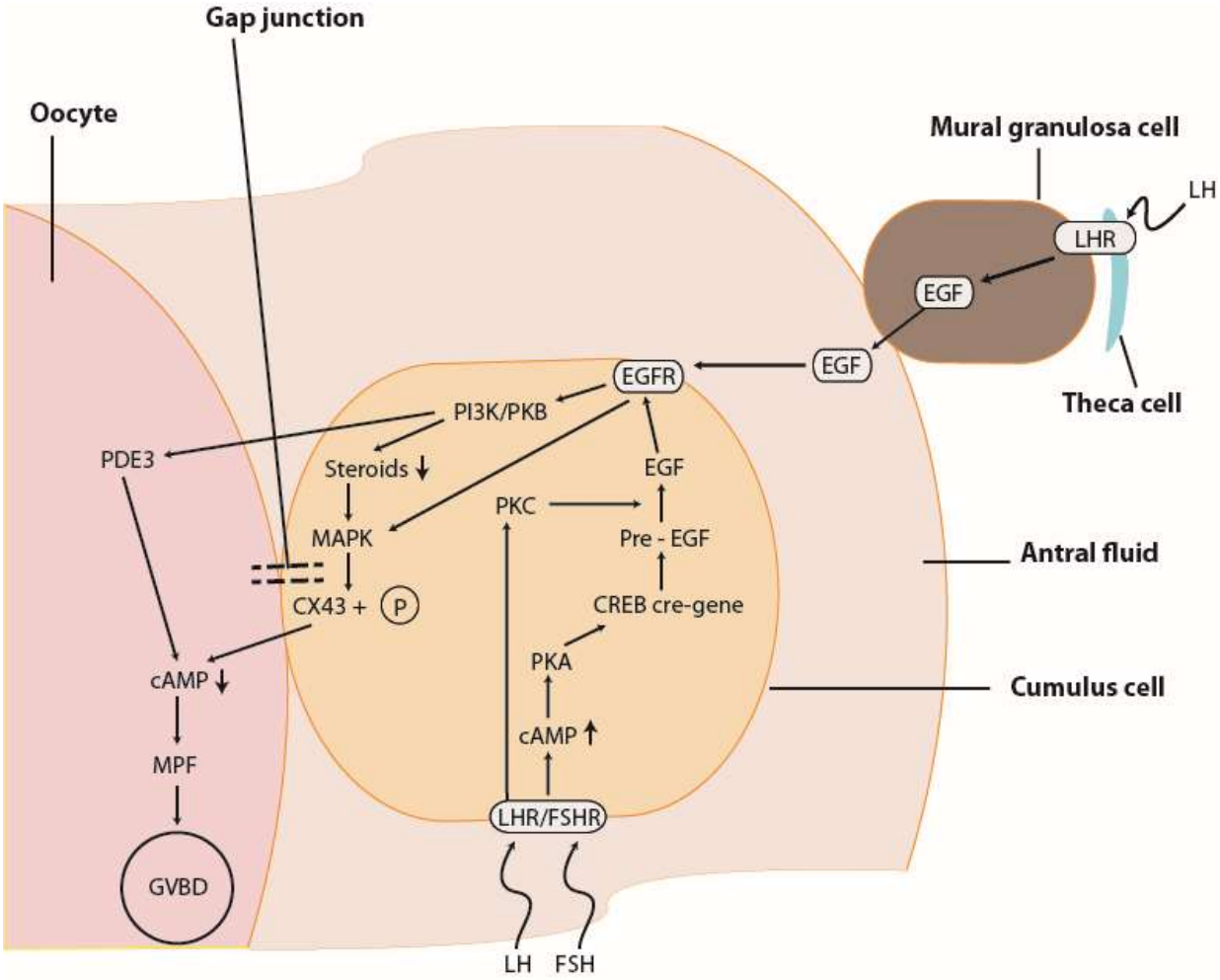

Figure 1.3: A proposed model for gonadotrophins-induced mammalian oocyte meiotic resumption in pre-ovulatory follicle. Abbreviations: LH, luteinizing hormone; LHR, luteinizing hormone receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PDE3, oocyte-specific phosphodiesterase 3; PKC, Protein kinase C; MAPK, mitogen activated protein kinase, CX43, connexin 43; CREB, cAMP-responsive element-binding protein, PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; MPF, maturation promoting factor; FSH, follicle stimulating hormone; FSHR, follicle stimulating hormone receptor; and GVBD, germinal vesicle breakdown stage. Circled P with + indicates to phosphorylation

1.2. In Vitro Fertilization

The fusion of the male and female gametes is known as fertilization. The process involves the combining of an oocyte and a sperm, which results in the formation of a single diploid cell or zygote from which a new individual organism will progress. There are several steps that lead to successful fertilization *in vivo* and *in vitro*. These steps are sperm capacitation, sperm oocyte binding, acrosome reaction and oocyte plasma membrane fusion.

Firstly, some modifications must be made to the sperm. Sperm capacitation (Figure 1.4) may be considered as a sequence of biochemical modifications that eventually enable the sperm to attach to the ZP and complete acrosome exocytosis. It includes the loss of seminal plasma proteins, but above all, the cholesterol is removed from the sperm membrane by the serum albumin. As a result, the membrane fluidity will rise, surface proteins required for oocyte contacts will be exposed, and the trans-membrane flow of signaling molecules will be enhanced (MANJUNATH and THERIEN, 2002, BAILEY, 2010, STIVAL et al., 2016). The permeability of the sperm membrane induces Ca^{2+} entry through sperm-specific Ca^{2+} channels (cation channels of sperm; CatSper) and HCO_{3-} ions by the Na^{+}/HCO_{3-} co-transporter (NBC). Intracellular Na^{+} will decrease, the K^{+} channels will be activated and the potential of hydrogen (pH) increases. The decrease of Na^{+} and K^{+} channel activation will lead to the hyper-polarisation of plasma membrane potential (ESCOFFIER et al., 2012, RITAGLIATI et al., 2018). The cAMP levels are enhanced by the activation of soluble adenylyl cyclase (sACY), protein kinase A activation, and by the phosphorylation of tyrosine proteins. These reactions engage numerous pathways in the sperm which cause all capacitation processes such as hyperactivated motility, ZP binding and acrosomal exocytosis preparation (BAILEY, 2010, ROBLES-GÓMEZ et al., 2021).

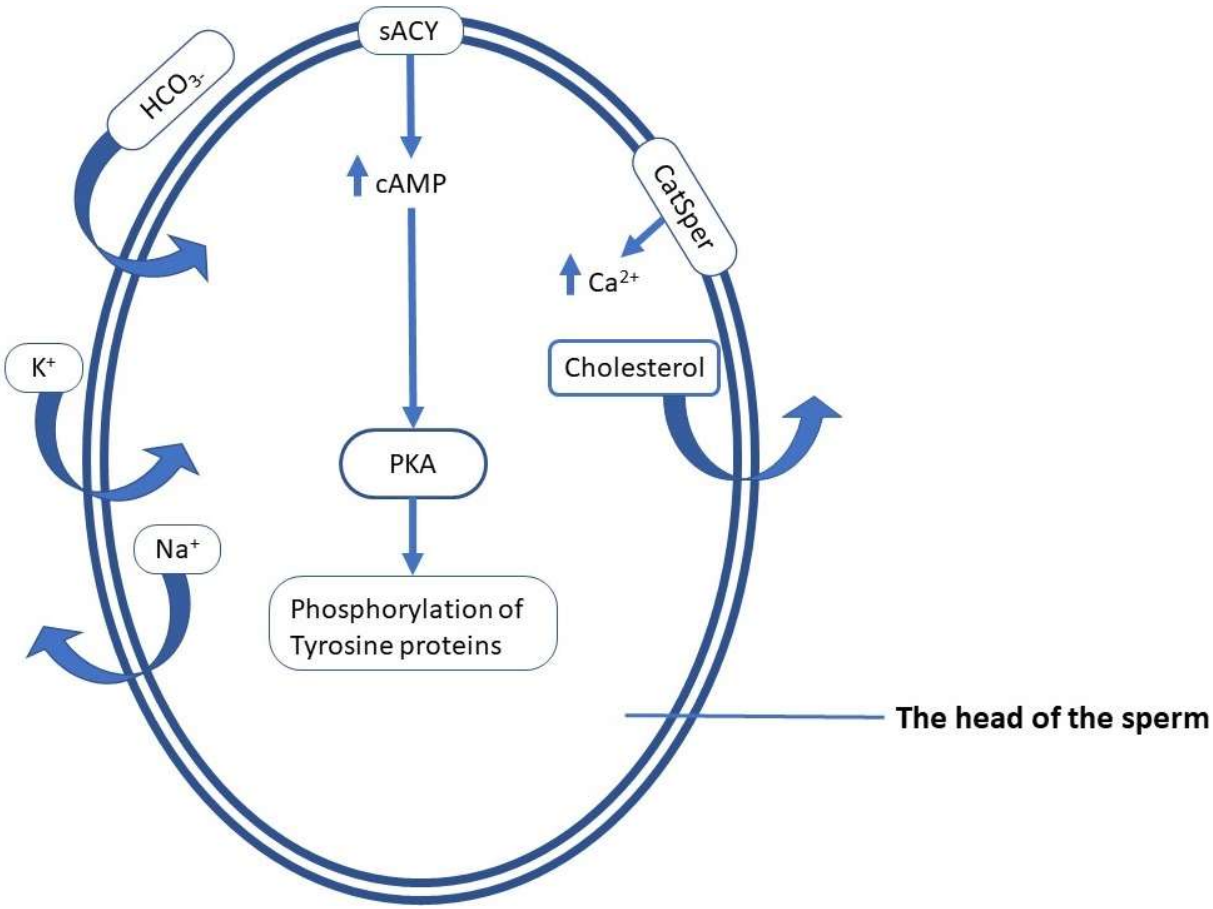

Figure 1.4: Sperm capacitation. This figure illustrates the factors that can effect sperm capacitation. The capacitation takes place in the head of the sperm. Abbreviations: sACY, soluble adenylyl cyclase; HCO3-, bicarbonate; CatSper, cation channels of sperm; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; K^+, potassium; Na^+, sodium.

To accomplish fertilization, sperm are generally considered to attach to the extracellular layer, which represents the primary barrier and the second level of contact between sperm and oocyte. The mammalian oocyte ZP layer includes up to four unique ZP proteins (ZP1-4). The ZP3α is the main zona pellucida protein that is responsible for the sperm-oocyte binding in pig (HARDY and GARBERS, 1994), but there is an evidence that ZP4 is responsible as well. The the purified ZP4 showed sperm binding activity, whereas the purified ZP3 did not, which indicated that ZP4 alone can bind to sperm (YONEZAWA et al., 2012). The acrosome reaction has a number of consequences in sperm including: protein G activation, membrane hyperpolarization, $Ca2^{+}$ activation and intracellular Ca^{2+} and pH increase (WASSARMAN et al., 2001). The next step involves the fusing of both sperm plasma membrane and the outer acrosomal membrane of the sperm through zona binding region (ZP3/ZP4) (YONEZAWA et al., 2012). ZP degradation is mostly caused by the hydrolytic action of enzymes, particularly acrosine, released from the acrosome. In addition, the motility of the sperm enables the sperm to enter the ZP (ABOU-HAILA and TULSIANI, 2000). At the same time, the cortical granules (CGs) in the oocyte will redistribute beneath the plasma membrane to release its contents to the perivitelline space (GOMEZ-ELIAS et al., 2020). This will occur to complete the sperm fusion.

1.3. Embryo Culturing

The quality and number of porcine embryos produced *in vitro* depend on *in vitro* culture environment (CHEONG et al., 2015). Studies to improve the composition of the culture media and to maximize the environment in culture have been done for many years. Culture media should contain minerals, proteins, energy (as glucose), lactate, calcium, amino acids, and pyruvic acid (PONIEDZIAŁEK-KEMPNY, 2020). Bovine serum albumin or fetal calf serum are the most often supplied proteins. Most current used pig embryo culture media are NCSU-23, Beltsville Embryo Culture Medium (BECM), modified Whitten's Medium (mWM) and Pig Zygote Medium (PZM) (FOWLER et al., 2018). Adding some supplements to culture media can be necessary for successful culturing to occur. One supplement that can be used in the media is phenazine ethosulfate that can increase the glucose level in the blastocyst and decrease the lipid as triglyceride that is a little harmful for pig embryos (ROMEK et al., 2011). Another supplement is glycine in pig embryo development that has shown a reduced rate of apoptosis in

embryos grown in this compound compared to embryos generated without this supplement (LI et al., 2018). In the presence of glycine, blastocyst cell numbers increased, especially in the trophectoderm lineage. Glycine is a precursor to the production of protein and nucleic acid that affects rapid proliferation of the cells (REDEL et al., 2016). Vascular endothelial growth factor (VEGF) is another important component in pig embryo culture media to improve their capacity for development (BISWAS et al., 2018). In pig embryo culture media, glucose and glutamines are widely applied as energy sources. Glucose metabolism through the pentose phosphate pathway is associated with increases in reactive oxygen species, the high concentration of glucose in NCSU-23 has been effectively substituted with pyruvate and lactate as alternative forms of energy (FOWLER et al., 2018).

1.4. Challenges in *In Vitro* Embryo Production (IVEP)

The production of embryos *in vitro* consists of three steps: *in vitro* maturation of oocytes (IVM), *in vitro* fertilization (IVF) of matured oocytes, and *in vitro* culture (IVC) of presumptive zygotes. While the IVEP technique reached an accepted success level in bovine in the 1980s (GRUPEN, 2014), the success of IVEP in porcine was still low (DANG-NGUYEN et al., 2011). In porcine, the IVEP procedures still face numerous issues that are still unsolved today including: inadequate cytoplasmic maturation, small percentages of embryo development, and fertilization irregularities such as polyspermy. Each step in the IVEP procedure has problems leading to IVEP inefficiencies. These problems will be described in more details in the following sections.

1.4.1. Challenges in *In Vitro* Maturation (IVM) of Oocytes

Several factors can affect IVM success such as: follicle size, cytoplasmic-nuclear maturation and the age of the animal providing the oocytes (GRUPEN, 2014). Oocytes retrieved from large follicles (>8 mm) require only 18 hours to complete oocyte maturation *in vitro* and their developmental competence is significantly greater than the oocytes obtained from medium sized follicles (3-7 mm) (KWAK et al., 2014). Moreover, the cytoplasmic and nuclear maturation of the oocyte must occur simultaneously to achieve full maturation of oocytes

(SCHOEVERS et al., 2005). Although nuclear and cytoplasmic maturation start at the same time, the nuclear maturation could be completed before the cytoplasmic maturation in *in vitro* conditions (GRUPEN, 2014). Unfortunately, the main problem of porcine IVM is a slow cytoplasmic maturation, which includes all the cytoplasmic modifications happening during the activity of meiosis, that could be stopped before completion at the end of the nuclear maturation. It is common for oocytes to be harvested from the ovaries after slaughter of animals from various ages, estrous cycle phases, and follicular size. As a result, these oocytes have been through various stages of development. Because of this, some oocytes may begin meiosis earlier than others during IVM, perhaps due to the imbalance between nuclear and cytoplasmic maturation rates inside the cell (FUNAHASHI et al., 1997, GILCHRIST and THOMPSON, 2007). Cytoplasmic maturation is thought to be the major factor affecting the maturation rate of oocytes (DANG-NGUYEN et al., 2011). Another issue for cytoplasmic maturation is that there are no markers to determine the stage of cytoplasmic maturation (DANG-NGUYEN et al., 2011). In addition, the age of the animal providing the source of oocytes can influence the IVM process of the oocytes. More embryonic developmental was observed in oocytes collected from sows ovaries (57% of blastocyst for day 7) compared to oocytes collected from younger animals (37.9% of blastocyst for day 7) (GRUPEN et al., 2003). Finally, the maturation media is not adequate enough for the oocyte maturation *in vitro*. The poor competence of oocytes using *in vitro* maturation is associated with maturation media and the culture environment applied in IVM (COGNIE et al., 2003, LIN et al., 2021). Accordingly, improvements in the maturation media are still needed to increase the rates of IVM and to achieve high quality embryos (FAN et al., 2017).

1.4.2. Challenges in *In Vitro* Fertilization (IVF)

The main problem affecting IVF is polyspermy (ABEYDEERA, 2002). Polyspermy is the main cause of embryo mosaicism (XIA, 2013). There are many factors causing a high polyspermy rate such as cortical granules (CGs) distribution, slow release of CGs, and the media used for fertilization. The high density of CGs leads to a reduction in polyspermy (ROMAR et al., 2005) by increased releasing of their contents. The content of CGs helps to block additional sperm penetration after the penetration of the first sperm cell. Polyspermy increased by the long duration of fertilization and the concentration of semen used in fertilization (DANG-NGUYEN

et al., 2011). Unfortunately, decreasing the number of sperm for each oocyte is not a useful way to reduce polyspermy; it could potentially decrease the overall penetration rate (GRUPEN, 2014). Another factor affecting polyspermy is the composition of IVF media (ABEYDEERA, 2002). The composition of fertilization media does not mimic the same environment as *in vivo*. Some additions into media can help to reduce polyspermy such as hyaluronan and osteopontin. Hyaluronan is a main glycosaminoglycan that exists in oviductal fluid, and it can reduce polyspermy by reducing sperm binding (NAGAI et al., 2006). Osteopontin can reduce the polyspermy by decreasing the amount of sperm attached to the ZP or strengthening the ZP (HAO et al., 2006). Finally, polyspermy can be affected by age of the animal providing oocytes. For example, oocytes derived from sows had lower polyspermic fertilization (16%) than oocytes derived from prepubertal gilts (31%) (MARCHAL et al., 2001).

1.4.3. Challenges in *In Vitro Culture* (IVC)

While the development of embryos into morula and blastocyst stages is more than 70 percent successful *in vivo*, studies show a much lower success rate of embryo development with *in vitro* matured oocytes (FOWLER et al., 2018). The main factor affecting IVC is the medium, because it does not fully replicate the characteristics found in the *in vivo* environment (GRUPEN, 2014). A medium that contains high-energy concentrations can be used to sustain the development of embryos such as North Carolina State University (NCSU-23) medium (BEEBE et al., 2007). It was recommended that embryo culture media be adjusted to mimic the composition of oviductal fluids (GRUPEN, 2014). Unfortunately, these media are not chemically specified to sustain embryo development from the fertilization point to the hatching stage of blastocysts. One major difficulty is that the embryonic culture media may be quite different from the *in vivo* conditions when comparing components. For example, NCSU-23 includes glucose as a source of energy for the development of embryos, but at a level around 32 times greater than that shown *in vivo* (STURMEY and LEESE, 2003). This increasing of glucose is related with an increase in reactive oxygen species which has a negative effect on embryos (FOWLER et al., 2018). Adding some substances like melatonin in cultural medium can improve the quality and the cleavage of embryos significantly (FERNANDO and ROMBAUTS, 2014). The effect of melatonin on embryos is to prevent reactive oxygen species (ROS) (TAMURA et al., 2012) that

can destroy proteins, lipids, nucleic acids, DNA, and RNA in embryos (GUPTA et al., 2010). Thus, the culture medium and its additives can affect embryo development.

1.5. Pre-Selection of Oocytes and Oocyte Maturation

In vitro maturation of oocytes (IVM) is an important step in completing *in vitro* production (IVEP) of embryos. Blastocyst rates are still low (less than 30%) after IVM and IVF procedures for swine (MARTINEZ et al., 2015a). The synchronization of cytoplasmic-nuclear maturation is important to achieve full maturation of oocytes (SCHOEVERS et al., 2005). Insufficient cytoplasmic maturation is the main reason for low efficiency of IVEP of embryos in porcine (ZHANG et al., 2012). Cytoplasmic maturation of an oocyte can be followed by redistribution of organelles, changes in cytoskeleton, and changes in the metabolism of cells (CAO et al., 2017, GE et al., 2008). The difference between immature and matured oocytes includes the redistribution of the cortical granules to the cortical zone beneath the oolemma in mature oocytes (KULUS et al., 2020), mitochondria in the center of ooplasm (KIM et al., 2016), and lipid droplets beneath the oolemma (KERE et al., 2020). The oocytes that are *in vitro* matured are usually less competent than matured oocytes obtained *in vivo* (ELAHI et al., 2016). The competency of matured oocytes can be determined by using many measures such as lipid metabolism, carbohydrate metabolism, redistribution of the mitochondria and mitochondrial function, reduction of oxygen radicals, epigenetic changes, growth factor secretions of the oocyte such as insulin-like growth factor-1 and epidermal growth factor (READER et al., 2015, KRISHER, 2013), and changes in the connection between cumulus cells and the oocytes through gap junctions (APPELTANT et al., 2015). There is no indicator of cytoplasmic maturation status for live cells.

Changes at the molecular and ultrastructural levels occur in oocytes during cytoplasmic maturation to support oocyte maturation competency (PAWLAK et al., 2012). To reach this competency, oocytes accumulate proteins and mRNA through maturation (YUAN et al., 2016), endoplasmic reticulum can be changed by redistributing into the cortex (ZHAO et al., 2017), and intracellular glutathione (GSH) levels will be increased (ZHIQIANG FAN, 2017). Another change to reach cytoplasmic maturation and oocyte competence is mitochondria redistribution which are the main producers of energy (YU et al., 2010). The low number of mitochondria is

reported to be linked to fertilization failure and embryo abnormality (BENKHALIFA et al., 2014, CHAPPEL, 2013). In addition, the distribution of mitochondria changes from the peripheral area of the oolemma to the inner area of the oocyte, around the nucleus, during maturation (VIET LINH et al., 2013). In fact, these distributions can be classified as peripheral distribution where all mitochondria are distributed beneath the oolemma; semi-peripheral where some of mitochondria are distributed under the oolemma and others distributed within the cytoplasm around the nucleus; and diffuse distribution characterized as all mitochondria distributed around the nucleus area (YANG et al., 2010, KATAYAMA et al., 2006). In mature oocytes (during MII stage), most of the mitochondria are in the diffuse pattern, and immature oocytes are mostly within the peripheral pattern (SUN et al., 2001). Another organelle, the CG also show different patterns of distribution depending on maturation. These patterns are specifically two patterns which are peripheral distribution, with a thin monolayer beneath the oolemma, and cortical distribution with a thick layer in the cortex of oocyte (PAWLAK et al., 2012). The peripheral pattern of CG is characterized for matured oocyte in cytoplasmic level, and immature oocytes usually have cortical distribution.

In summary, some organelles like mitochondria and cortical granules have patterns allowing recognition of matured oocytes versus immature oocytes that can be visualized by fluorescent markers. MitoTrackers and Rhodamine-123 are fluorescence dyes used in many studies to follow the mitochondrial distribution through oocyte maturation (SUGIMURA et al., 2010, JOHNSON et al., 1980, YAMOCHI et al., 2016, ABDOON et al., 2011, DE LOS REYES et al., 2011, FERNANDES et al., 2012, LEONI et al., 2015, NIU et al., 2015). These dyes can be used with high viability of oocytes if they are used at optimum concentrations. Mitochondrial distribution using MitoTracker as a marker for oocyte maturity has been followed by many species such as camel (ABDOON et al., 2011), dog (DE LOS REYES et al., 2011), mouse (FERNANDES et al., 2012), sheep (LEONI et al., 2015), and pig (NIU et al., 2015). In addition, many dyes can detect CG distribution, especially lectins such as peanut agglutinin (PNA) conjugated with fluorescence dyes like fluorescein isothiocyanate (FITC) (COY et al., 2002). Cortical granule distribution throughout maturation of oocytes using fluorescence dyes was reported by many researchers (ZHENG et al., 2016, GHETLER et al., 2006, BELLO et al., 2016, ANDREU-VAZQUEZ et al., 2010, DIAZ and ESPONDA, 2004). All previous studies were done using fixed oocytes to determine mitochondrial and cortical granules distribution. The pre-

selection of oocytes using these dyes to segregate matured or immature oocytes may be a useful method to improve fertilization rate and embryo quality.

1.6. Effect of Maturation Media on Oocyte Maturation

Generally, pig oocytes obtained *in vitro* have less developmental competence than those developed *in vivo*, and this suggests that the present method of IVEP is inadequate. This issue usually represents a cytoplasmic maturation fault in an oocyte that could be related to the inadequate *in vitro* maturation in comparison to the *in vivo* maturation (HYUN et al., 2003). For each developmental stage, media needs to be specific. The use of chemically defined medium allows an investigation of the influence of the different components necessary for the proper growth of embryos (FOWLER et al., 2018). Developing an oocyte maturation medium could be a critical and crucial step towards achieving nuclear and cytoplasmic oocyte maturation and coordinating those activities (COMBELLES et al., 2002). This is extremely necessary as the morphology of the germ vesicle is much different when collecting oocytes (FUNAHASHI et al., 1997). Oocyte maturation medium might influence embryonic development, cell number of embryos and embryonic apoptosis (WATSON, 2007).

There are many factors that can affect maturation including steroids, oviductal fluid, follicular fluid, and gonadotropins (PYOOS et al., 2018), and the main factors in media that affect oocyte maturation may be anti-oxidant content, oxygen tension, and some supplements or chemicals in media ingredients (NAGAI et al., 2006). Those supplements include, for example, fetal serum (FCS), gonadotropins (ABEYDEERA, 2002), porcine follicular fluid (pFF), and various hormones used as supplements in IVM media (ZHANG et al., 2012). Researchers have been working on developing IVM strategies for all previous factors or problems. They succeeded in several aspects, such as media supplements. First, the *in vitro* environment must support both nuclear and cytoplasmic maturation.

Three major kinds of IVM media are currently widely applied, such as tissue culture medium (TCM) - 199, mWM and NCSU23, as those offering the greatest oocyte maturation competence. Although the major components of these media are similar with some modifications, it is obvious that even very minor changes in the component concentrations might influence the outcomes (FOWLER et al., 2018). There is a diversity of maturation media

that exist, and it is extremely challenging to choose the basic media for IVM. Another example of maturation media that used is modified Tyrode's medium containing lactate and pyruvate (mTLP) with pFF or serum (ZHANG et al., 2012). In point of fact, pFF was found to improve the nuclear and cytoplasmic maturation of oocytes (GRUPEN, 2014). Many researchers suggest adding other ingredients to the media to reproduce maturation conditions. Adding porcine leukemia inhibitory factor (pLIF) with a concentration of 1% (v/v), for example, improved oocyte maturation by activating its maturation processor (DANG-NGUYEN et al., 2014). Moreover, growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and growth differentiation factor 9 (GDF-9) improved oocyte maturation (ZHANG et al., 2012). The maturation media should supply the oocytes with components of the glycolytic pathway like adenosine triphosphate (ATP) and pyruvate (DE ARAUJO et al., 2009). To increase the maturation of immature oocytes, adding supplements to standard media seems to be important. To achieve maximum maturation of pig oocytes, epidermal growth factor, essential or nonessential amino acids and gonadotropins are also important to use (KIM et al., 2011).

1.7. Vitrification of Embryos and Embryo Transfer

Vitrification is the process of solidification of a liquid without ice-crystal formation by decreasing the temperature rapidly and increasing the viscosity of the solution (YAVIN and ARAV, 2007). Vitrification may avoid intracellular and extracellular modifications and damaging the cells caused by slow-freezing such as damaging mitochondria, DNA, plasma membrane, and nuclei (DOBRINSKY and JOHNSON, 1994). The success of vitrification depends on many factors: cooling rate, the concentration and behaviour of cryoprotectants and other additives to the media (viscosity), as well as sample volume (SARAGUSTY and ARAV, 2011). Increasing cooling rate from about 20,000 ºC/minute to 135,000 ºC/minute resulted in an increase of 33.9% in survival rate of porcine blastocysts after warming (BEEBE et al., 2005). The concentration of cryoprotectant affects survival rate of porcine blastocysts. The concentration of 16.5% of dimethylsulfoxide plus 16.5% ethylene glycol achieved 67% survival rate, but it was not significantly higher than that achieved with a concentration of 20% dimethylsulfoxide plus 20% ethylene glycol (57% survival rate) (BERTHELOT et al., 2007). Decreasing sample volume also caused increases in cooling rate and increases in vitrification

efficiency (SARAGUSTY and ARAV, 2011). Unfortunately, vitrification affects piglet development in ET procedures. After ET, gilts that received vitrified embryos gave birth to 12.3% piglets in comparison with gilts that received fresh embryos (68.8%) (MISUMI et al., 2003). The low percentage of piglets derived from vitrified embryos may be caused by lack of selection of the high-quality matured oocytes or high-quality embryos.

1.7.1. Challenges in Vitrification of Embryos

Vitrification of pig embryos is not an optimized technique, and damage associated with cryopreservation interferes with the development rate of morulae into blastocysts (SARAGUSTY and ARAV, 2011, MANDAWALA et al., 2016). In addition, a higher number of vitrified-warmed embryos is required for ET compared to fresh embryos without vitrification (RIHA and VEJNAR, 2004). Other problems occur with vitrification-warming procedures where there is a lower survival rate of embryos after this procedure than with fresh embryos (BERTHELOT et al., 2007, MANDAWALA et al., 2016). The vitrified embryos also have slower growth rate or development than the fresh embryos (BERTHELOT et al., 2007, SARAGUSTY and ARAV, 2011, MANDAWALA et al., 2016, NOHALEZ et al., 2018a). Moreover, the quality of vitrified-warmed embryos is less than fresh embryos as measured by the number of blastocyst cells (KAMOSHITA et al., 2017, UCHIKURA et al., 2016).

There are many factors that influence a successful vitrification-warming procedure. Type and concentration of cryoprotectant are the major factors that affect the vitrification procedure (VH et al., 2014). Several studies considered the importance of the cryoprotectant type, and researchers have tried to achieve a high post-warm outcome. There are two main categories of cryoprotectants; penetrating and non-penetrating cryoprotectants (BARTOLAC et al., 2018). The penetrating cryoprotectants include glycerol, dimethylsulfoxide (DMSO), and other polyols such as 1,2-propanediol (PROH) and 1,2-ethandiol (ethylene glycol, EG) (SZUREK and EROGLU, 2011). The non-penetrating cryoprotectants are macromolecules such as trehalose, sucrose, ficoll, and raffinose (BARTOLAC et al., 2018). Using different concentrations of the same cryoprotectant affects positively or negatively the vitrification outcome. For example, using carboxylated ε-poly-L-lysine (COOH-PLL) as cryoprotectant in different concentrations (1%, 10%, 20%, and 30%) leads to improved vitrification outcome with concentration increasing up to

20% (KAMOSHITA et al., 2017). On the other hand, the pH in vitrification media can successfully affect vitrification-warming procedures. The pH should be stable through the procedure at 7.2–7.4. To reach this level, researchers have tried to use CO_2 incubators to calibrate the pH, or a specific media that is pH-stable (CUELLO et al., 2016). Other limitations for using vitrification are the procedure duration and embryo handling (OCHOTA et al., 2017). The contamination of liquid nitrogen or equipment used in vitrification is another factor that influences the procedure outcome (KADER et al., 2009, MANDAWALA et al., 2016, VH et al., 2014). In pig embryos, there is another limitation in use of vitrification procedures. This factor is related to the high level of lipid droplets in oocytes or embryos in comparison to other species like ovine, bovine (GAJDA, 2009), and mouse (about 6.8 times more than mouse) (HUANG et al., 2019). The high level of lipid makes the pig embryos more sensitive to cooling procedures (HUANG et al., 2019, KAMOSHITA et al., 2017, NOHALEZ et al., 2018a).

1.7.2. Challenges in Embryo Transfer

Even though pigs have been born following ET using embryos and blastocysts developed *in vitro*, the percentage of success is still low (HWANG et al., 2016). Even though, the successful embryo transfer in porcine results low pregnancy and survival rates (35-40%) of piglets from transferred embryos (YOUNGS, 2001). There are various explanations or factors proposed related to the inability to develop transferred cleaved embryos.

Pigs have unique mechanisms for detecting pregnancy, so large numbers of transferred embryos are normally needed to achieve a successful conception (KING and DE SOUSA, 2006). However, the problem of using large number of transferred embryos occurs if most of embryos successfully undergo implantation, and then override the capacitation in uterus. It is possible that just a few numbers of qualified embryos are available for transfer on the process day. Vitrified embryos can provide these large numbers of embryos. However, there are problems with using these embryos. After cryopreservation, vitrified morulae and early blastocysts exhibit microfilament abnormalities that decrease the developmental competence (DOBRINSKY et al., 2000). During vitrification of embryos, liquid nitrogen can damage the intracellular mechanisms as well as the organelles, and indeed the main cytoarchitecture of the embryo can be changed or destroyed (DOBRINSKY, 2001). Also, the high lipid content in pig oocytes and embryos

specifically make these embryos sensitive to the cryopreservation (SOMFAI et al., 2019). However, if there is a large number of transferred embryos and the survival rate is high, the crowding could lead to embryonic mortality (KING and DE SOUSA, 2006).

There is a lack of knowledge about the best timing between the developmental stage of the transferred embryos and the recipient's cycle (MARTINEZ et al., 2019). With morulae embryo transfers, pregnancy rate dropped significantly in comparison to blastocyst transfer perhaps because of the low developmental efficiency of morulae (HAZELEGER and KEMP, 1994). Morula embryos may not survive at this stage because the hormonal factors contributed with corpus luteum development and endometrial secretions in the uterine lumen are insufficient (ANGEL et al., 2014). Endometrium receptivity refers to the capacity of the endometrium to accept the cyclic changes for embryo implantation. An unbalanced estrogen-progesterone ratio can, however, reduce endometrium receptivity and increase the failure of the blastocyst implantation after ET to about 67% (FENG et al., 2021, YANG et al., 2020, LENSEN et al., 2016, PARRILLA et al., 2020). The lipid content differences in the endometrium can be an impact factor for the implantation success following embryo transfer (LI et al., 2019).

Another factor that can affect the ET performance are the recipients or the donors themselves. Sows selected for use as recipients should have had successive litters and a history with no reproductive or health issues, particularly during the last farrowing and lactating phase (CAMERON et al., 1989). The breed of the recipient may be an important factor in pig ET. For example, Meishan gilts have been used effectively as recipients for their excellent survival rate of embryos with pregnancy rate – 86% and 9.3 live piglets per female (BRUSSOW et al., 2000). The age of donor is one factor that can also affect the embryo transfer success. The embryos derived from pre-pubertal gilts are less able to develop and survive than multicyclic (sow) pigs (YOUNGS, 2001).

Traditional surgical collection is used to collect and transfer embryos. Due to the cost of surgical procedures for both collection and transfer of the embryo, commercial opportunities for ET in the pig industry have been limited (YOSHIOKA et al., 2012). At this point, sterile surgical standards are sometimes difficult to meet in farm environments, and are characterised by a variety of logistical challenges and expenses for commercial transfers on farm (CAMERON et al., 2006). Therefore, surgical embryo transfer procedures have a limited use, due to their high cost, procedure time durations, , potential postoperative problems and long wound treatment

periods (WIECZOREK et al., 2015, MARTINEZ et al., 2017). Only two or three repetitions of surgical embryo collection can be done because of scar tissue formation. These problems are normal limitations of using surgeries (HAZELEGER and KEMP, 1999).

An alternative technique of embryo transfer is the non-surgical technique of endoscopy. Compared to surgical procedures, this method needs endoscopic equipment and professional expertise (BRUSSOW et al., 2000). Furthermore, the uterus might be damaged when the guide is inserted into the uterus very deeply (HIRAYAMA et al., 2020). The type of embryo catheter and its quality are extremely important. The ET catheter must be non-toxic to embryos, be bio-compatible and smooth enough just to avoid oviduct membrane, uterus and cervical injuries, but it should be elastic enough to expand through lumen of the uterus or oviduct along the natural pathway for the required lengths; it must also be as thin as possible, ensuring the transfer of embryos in a very short period of time (up to 2-4 minutes) with a minimum culture media amount (WIECZOREK et al., 2020). To prevent embryos from being deposited between the layers of the uterine wall, the catheter or A1 straw must be inserted into the lumen (CAMERON et al., 1989). With regard to developing non-surgical or endoscopic transfer procedures, it is important to determine the impact on the success rate that is being achieved at the uterine location to which embryos are being transferred (WALLENHORST and HOLTZ, 1999). Currently, there are still some problems with this endoscopic ET technique, like the ET catheter not passing into the cervix (MARTINEZ et al., 2016) as well as incorrect catheter location during ET (MARTINEZ et al., 2015b).

1.8. General Hypothesis and Objectives

1.8.1. General Hypothesis

Pre-selection of matured oocytes based on mitochondrial and cortical granules distribution as cytoplasmic maturation markers could improve the efficiency of *in vitro* production, vitrification, and embryo transfer procedures.

1.8.2. General Objectives

1. Develop a method to preselect matured oocytes *in vitro* using fluorescent dyes.
2. Improve *in vitro* production of embryos using preselected oocytes.
3. Determine the preferred cortical granule and mitochondrial distribution of oocytes using different maturation media.
4. Preserve embryos following preselection of oocytes using vitrification technique and embryo transfer.

CHAPTER 2: VIABILITY OF PIG OOCYTES STAINED WITH FLUORESCENT DYES FOR ASSESSMENT OF MITOCHONDRIAL AND CORTICAL GRANULE DISTRIBUTION

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Relationship of this study to the dissertation

In chapter 2, we evaluated the viability of oocytes using fluorescent dyes (MitoTracker orange, MitoTracker green, Rhodamine-123, peanut agglutinin, and wheat germ agglutinin) using wide-field fluorescence microscopy and confocal microscopy. We evaluated the distributions of mitochondria and cortical granules before and after maturation (this method will be used in chapters 3, 4, and 5). Our hypothesis for this study was the central pattern (diffuse) mitochondria and peripheral cortical granules distribution, indicators of oocyte maturity, can be visualized in live oocytes with high viability.

Authors' Contributions

Hamza Al-Maamory: Performed the study, participated in experimental design, wrote the manuscript.
Carl Lessard: Supervised, and participated in the experimental design and critical revision of the manuscript.
Dinesh Dadarwal: Provided critical notes about Imaris software and critical notes to revise the manuscript.

2.1. Abstract

Insufficient cytoplasmic maturation of pig oocytes could be one of the main reasons resulting in a low efficiency of *in vitro* production of embryos (IVEP). The distribution patterns of mitochondria (MT) and cortical granules (CGs) have been associated with the cytoplasmic maturation of oocytes. MT and CGs distributions can be observed using MitoTracker and Peanut Agglutinin fluorescent dyes. It was hypothesized that MT and CG distribution of pig oocytes can be assessed using fluorescent dyes, and MT or CG distribution are better visualized in adult sows than prepubertal gilts. The specific objectives of this study were to evaluate: 1) appropriate concentrations of fluorescent dyes (MitoTracker orange, MitoTracker green, Rhodamine 123, Peanut Agglutinin and Wheat Germ Agglutinin) and viability on pig oocytes; and 2) the differences in distribution of MT and CGs in oocytes harvested from pre-pubertal and adult sows before and after maturation.

Grade 1 and 2 oocytes were harvested from slaughtered pre-pubertal gilts or sows. Oocytes were matured *in vitro*, cumulus cells were denuded before the staining with different concentration of fluorescent dyes i.e. MitoTracker orange (MTO), MitoTracker green (MTG) and Rhodamine-123 (RH-123) for MT distribution, and Peanut agglutinin (PNA) and Wheat germ agglutinin (WGA) for CGs distribution. The stained oocytes were observed under wide-field fluorescence (Experiment 1 and 2) and confocal microscopes for confirmation of MT and CG distributions (Experiment 2). The distribution (diffused pattern: matured oocytes) of MT in oocytes, after 44 hrs of maturation, did not differ significantly between oocytes from prepubertal gilts (48% ± 18.5; 64/135) and adult sow (61% ± 10.2; 38/62). However, distribution of CGs (peripheral pattern) was different due to source of pigs ($P \leq 0.05$); prepubertal oocytes had less (28% ± 9.5; 40/148) oocytes with a peripheral CGs (matured oocytes) than the adult sows (69% ± 13.1; 42/62). In this study, we chose 200 nM MitoTracker green and 625 nM PNA as the optimal concentrations for observation with wide-field microscopy to obtain higher viability of oocytes. In summary, this study demonstrated that pig oocytes can be stained with fluorescent dyes and sorted as patent oocytes.

2.2. Introduction:

In vitro maturation (IVM) of oocytes is an important step for successful *in vitro* production of embryos (IVEP). Oocyte competence includes nuclear and cytoplasmic maturation. Nuclear maturation is the step during meiosis process to change from prophase I to metaphase II (MII). Cytoplasmic maturation refers to the changes which prepare the oocyte for fertilization. It includes the uniform distributions of MT throughout the cytoplasm and CGs near the oolemma. Insufficient cytoplasmic maturation is the main reason for low efficiency of IVEP in porcine. Oocyte maturation can be evaluated by its overall morphology and staining methods.

Morphological characteristics such as MT and CG distribution, or zona pellucida shape determine cytoplasmic maturation to select high-quality oocytes for successful *in vitro* fertilization (IVF) for embryo production. Different staining techniques are available to assess oocyte maturation using fluorescent dyes. MitoTracker and Rhodamine-123 are fluorescence dyes used in many studies to follow the MT distribution through oocyte maturation (SUGIMURA et al., 2010, JOHNSON et al., 1980, YAMOCHI et al., 2016, ABDOON et al., 2011, DE LOS REYES et al., 2011, FERNANDES et al., 2012, LEONI et al., 2015, NIU et al., 2015). These dyes can be used without oocyte death if used in optimum concentrations. MT distribution using MitoTracker, as a marker for oocyte maturation, has been used in different species such as camel (ABDOON et al., 2011), dog (DE LOS REYES et al., 2011), mouse (FERNANDES et al., 2012), sheep (LEONI et al., 2015) and pig (NIU et al., 2015). In addition, many dyes can detect CGs distribution, especially lectins such as PNA conjugated with fluorescein isothiocyanate (PNA-FITC) (COY et al., 2002). Assessment of CGs distribution throughout the maturation phase of oocytes using fluorescence dyes has been previously reported by many researchers (ZHENG et al., 2016, GHETLER et al., 2006, BELLO et al., 2016, ANDREU-VAZQUEZ et al., 2010, DIAZ and ESPONDA, 2004). All previous studies were done using fixed oocytes to determine MT and CG distributions.

Mitochondria perform significant functions in energy and Ca^{2+} production during oocyte development (DUMOLLARD et al., 2009) for both nuclear and cytoplasmic maturation (KRISHER et al., 2007). MT distribution is an indicator for cytoplasmic maturation. In porcine, the distribution of MT changes from peripheral to the central area during the maturation process (VIET LINH et al., 2013). Three patterns of MT distributions are expressed at different stages of

maturity i.e. peripheral, semi peripheral, and diffuse (YANG et al., 2010). The diffuse pattern represents the MT distribution around the nucleus (KATAYAMA et al., 2006). In mature oocytes (MII stage), most of MT re-distribute to the center of oocyte (SUN et al., 2001). RH-123 is another dye (SUGIMURA et al., 2010) that stains the MT without using carriers or expressing any cytotoxic effects (JOHNSON et al., 1980).

Another dye which can be used for evaluation of MT distribution is MitoTracker. There are many kinds of MitoTracker such as red, deep-red, green, orange, and blue. MitoTracker green (MTG) is a dye that stains live MT selectively with no or low toxicity (CRUZ et al., 2011). The MTG can be used to determine the MT distribution as a marker for oocyte maturity in many species using fixed oocytes from bovine (ABDULHASAN et al., 2017), murine (MOAWAD et al., 2014), and porcine (ALVAREZ et al., 2016).

Cortical granules, the derivative of the Golgi apparatus (HOODBHOY and TALBOT, 2001), are found in the cortex of oocytes before fertilization (LIU, 2011). CGs usually have a different distribution during oocyte maturation (FERREIRA et al., 2009), and CG distribution is considered an important indicator of oocyte maturation (MIYARA et al., 2003). There are two specific patterns of CGs distribution in porcine oocytes: peripheral distribution under oolemma for mature oocytes and cortical distribution for immature oocytes in the cortex (PAWLAK et al., 2012). There is another pattern of CGs distribution in maturing oocytes, intermediate, some of CGs in peripheral pattern and some in cortical pattern (APPARICIO et al., 2011, SUZUKI and SAITO, 2006). The other lectin-based dye is WGA that can be used to determine CGs distribution (BURKART et al., 2012) through binding to cortical granule glycoconjugates such as α-D-acetylgalactosamine, galactose, N-acetylglucosamine, N-acetyllactosamine, N-acetylneuraminic acid, fucose and D-N-acetylgalactosamine (LIU, 2011). It also binds with the external and internal layers of the zona pellucida (MAYMON et al. (1994). In porcine, WGA can be used to determine oocyte maturation through staining of the zona pellucida (TAKAHASHI et al., 2013). Another use of WGA to determine the integrity of the plasma membrane (BRAMS et al., 2014).

In this study, we hypothesized that MT and CG distribution can be assessed using fluorescent dyes, MT and CG distribution is better visualized in oocytes harvested from adult sows than prepubertal gilts, and sorted oocytes having MT or CG distribution associated with “matured” oocytes facilitate fertilization.

The specific objectives for this study were:

1. To evaluate the appropriate concentrations of fluorescent dyes (MTO, MTG, RH-123, PNA and WGA) on viability of pig oocytes.

2. To compare the MT and CGs distribution by wide-field fluorescence microscopy and confocal microscopy.

3. To compare the distribution of MT and CGs in oocytes from pre-pubertal gilts and adult sows.

2.3. Materials and Methods

2.3.1. Animals and Chemicals

This study was conducted using oocytes from prepubertal gilts and adult sows slaughtered at a local abattoir (Country Choice Meats, 81 km north of Saskatoon and Valley Meat Processors, 40 km north of Saskatoon, respectively). All chemicals used in this experiment were purchased from Sigma-Aldrich Canada Co. (Oakville, Ontario, Canada), unless otherwise mentioned. All media were filtered with a 0.22 μm PVDF filter (Argos technology, Elgin, USA) for this study.

2.3.2. Oocyte Collection and *In Vitro* Maturation

Ovaries were procured from abattoirs and transported to WestGen Research Suite, Western College of Veterinary, University of Saskatchewan at 35-37 ºC using thermo-container at the time of slaughtering and transporting, and washed (3X) with normal saline (0.9% NaCl) at room temperature (RT) before immersion into glucose solution (0.128 M), at RT for 30 min. Cumulus oocyte complexes (COCs) were collected from follicles (3-8 mm in diameter). Only grade 1 and grade 2 COCs (at least 3 layers of cumulus cells) were chosen for further maturation. The COCs were washed (3X) with washing medium (TCM-199, Invitrogen, Burlington, Canada; supplemented with 25 mM of HEPES and 50 mg/ml gentamycin) at 37 ˚C. The COCs were rinsed (1X) in maturation medium (TCM-199, 3.05 mM D-glucose, 0.1% polyvinyl alcohol, 0.91 mM sodium pyruvate, 50 mg/ml gentamycin supplemented with 0.57 mM L-cysteine, 10 ng/ml epidermal growth factor (EPG), 0.01 U/ml Follitropin (Bioniche, Oakville, Canada) and 5 μg/ ml luteinizing hormone (Lutropin-V®; Bioniche, Belleville, Canada)). Subsequently, COCs

(maximum 50 COCs per well) were transferred into a 4-well dish with 500 µl of maturation medium/well and incubated at 38.5 ˚C under a controlled environment (5% CO2, 5% O2 and 90% N2 in a humidified environment) for 44 hrs without mineral oil covering.

2.3.3. Experiment 1: Determination of Oocyte Viability and Optimal Concentration of Fluorescent Dyes.

In this study, the oocyte viability and suitable concentrations of fluorescence dyes i.e. MTO (M7510; ThermoFisher Scientific, Canada; Excitation: 554 nm and Emission: 576 nm), MTG (M7514; ThermoFisher Scientific; Excitation: 490 nm and Emission: 516 nm), RH-123 (R8004;Excitation: 511 nm and Emission: 534 nm), PNA-FITC (L7381; Excitation: 552 nm and Emission: 565 nm), and WGA (W834; ThermoFisher Scientific; Excitation: 495 nm and Emission: 519 nm) in live pig oocytes were evaluated.

After the IVM procedure described above (section 2.3.2), the cumulus cells surrounding COCs were denuded using 100 µl of 0.1% hyaluronidase in TCM-199 and repeated pipetting for 80-100 times, using a 200 µl pipette. The denuded oocytes were washed (2X) in washing buffer (phosphate buffered saline [PBS] + 0.1 % PVA). After washing, oocytes were transferred into 50 µl droplets of the specific fluorescent dye as follows:

1. MT distribution:
    a. MTO: 100, 200 or 350 nM
    b. MTG: 200, 250, 300, 350, 400, 450 or 500 nM
    c. Rh-123: 13, 26, 66 or 79 µM
2. CG distribution:
    a. PNA: 833.33 or 625 nM.
3. Plasma membrane integrity:
    a. WGA: 526, 789 nM or 1.3 µM
4. Viability:
    a. Propidium iodide (PI; P1304MP; 1 mg/ml; ThermoFisher Scientific) – in combination with MTG, RH-123 or PNA
    b. Fluorescein diacetate (FDA; F1303; 2.5 µg/ml; ThermoFisher Scientific) – in combination with MTO or WGA

The oocytes along with MT, CG and plasma membrane dyes were incubated at 38 ºC under 90% N_2, 5% CO_2, and 5% O_2 conditions for 30 min. After incubation, oocytes were washed (3X) in washing buffer and counterstained with PI for MTG, RH-123 and PNA stained oocytes, or with FDA for MTO and WGA-stained oocytes to evaluate their viability. Oocyte viability was checked at 0, 22, and 44 hr after washing the oocytes. After incubation, oocytes were transferred into 5 µl droplets of PI (1 mg/ml) or FDA (2.5 µg/ml), for live/dead counterstaining, and further incubated at 38 ºC under 90% N_2, 5% CO_2, and 5% O_2 conditions, for 7 min. Oocytes were washed (3X) with washing buffer, and then examined under a wide-field fluorescence microscope (Olympus SZX16, Japan) using GFPA Filter Set and 20X magnification. Petri dishes (35 mm) were used to contain oocytes with the washing buffer. Oocytes (90-120 oocytes/ concentration/ dye) emitting red fluorescence in PI counterstaining and green fluorescence in FDA counterstaining represented dead and live oocytes, respectively. The percentages of live/dead oocytes out of total were calculated in this experiment.

2.3.4. Experiment 2: Distribution of Mitochondria and Cortical Granules in Pig Oocytes

2.3.4.1. Staining of oocytes and *in vitro* maturation

The best concentrations of MTG (200 nM) and PNA (625 nM), based on Experiment 1, were used to determine MT and CGs distribution, respectively. Oocytes were harvested from pig ovaries and initially processes as described in Experiment 1. Before IVM, oocytes were divided into two groups. In group 1, oocytes (n= 90 for MTG and n=90 for PNA) were denuded using 100 µl of 0.1% hyaluronidase in TCM-199 and repeated pipetting for 80-100 times, using a 200 µl pipette. In group 2, oocytes (n=90 for MTG and n=90 for PNA) were first matured *in vitro* for 44 hrs and then denuded with hyaluronidase and repeated pipetting. The denuded oocytes were washed twice with PBS containing 0.1 % polyvinyl alcohol (PVA) and then transferred into 50 µl droplets of 0.1% PVA in PBS with 200 nM MTG or 625 nM PNA. The oocytes were further incubated at 38.5 ˚C under 90% N_2, 5% CO_2, and 5% O_2 conditions, for 30 min. The stained oocytes were washed (3X) with 0.1% PVA in PBS and transferred into the same medium without dye. Oocytes were observed under a wide-field fluorescence microscope (as in Experiment 1) and then under a confocal microscope to confirm MT and CG patterns. Under a wide-field

fluorescence microscope, the oocytes were segregated separately in 35 mm dish for each pattern. The stained oocytes were fixed with 4% paraformaldehyde, and then stored in the refrigerator until confocal examination.

In confocal microscopy, 15 oocytes of each pattern were used (45 oocytes for MT and 45 oocytes for CGs). Glass bottom dishes (35 mm) were used with five oocytes from the same pattern in each dish covered by mineral oil. Images were captured with a Carl Zeiss confocal microscope (710 LSM two-photon; Oberkochen, Germany) camera using a 512 x 512 pixel array and a 40X objective.

AutoQuant X3 software (Media Cybernetics, Rockville, MD, USA) was used for deconvolution of confocal images (with default settings for the theoretical point spread function to account for convolution along the z-axis). Imaris software 8.4 (Bitplane AG, Badenerstrasse, Zürich, Switzerland) was used to analyze MT and CGs distribution patterns. In Imaris, a surface was created manually to illustrate the plasma membrane of the oocyte. To visualize the illustrated ooplasmic volume of MT or CGs, the outside of oocyte surface was set in Imaris to the value of zero. To determine the size of spots representing MT and CGs staining intensities, the volume of MT or CGs was used with the following filters: estimated diameter set to 1 μm for MT and 0.2 μm for CGs. To determine the distance between the centers of spots, the extension of spot-to-spot distance was used. Within the distmin filter (Appendix A), the spots within a 2-μm distance were determined. These spots identified as clusters of MT using the volume filter. The spots with a volume of 2 μm^3 and within the distance of 2 μm were regarded as MT clusters, and the rest of the spots were considered as individual MT. The MT and CGs distribution was identified based on the distance-transformed channel created based on the surface created previously. The MT peripheral pattern was determined within 10 μm of the oolema and central pattern was designated for the rest. The CGs peripheral pattern was determined within 2 μm of the oolema and a central pattern designated for the rest.

2.3.4.2. Confirmation of mitochondrial distribution patterns

Oocytes (N=100) stained with MTG were first categorized under wide-field fluorescence microscopy. Four MT patterns were observed under wide-field fluorescence microscopy (Figures 2.1 and 2.2) as described previously (Lio, 2011; Brevini *et al*.,2005; Isom *et al*., 2012; Suzuki et

al., 2005): 1) diffuse pattern where the majority of MT were distributed in the central area of the cytoplasm, with no MT in the peripheral area; 2) semi peripheral pattern where some MT were distributed in the central area and some in the peripheral area; 3) peripheral pattern where the majority of MT were distributed in the peripheral area only; and 4) unclassified where the pattern did not be recognized since the poor signal of MTG. These MT patterns were confirmed with confocal microscopy. The threshold area determined as central or peripheral area was 10-µm distance from peripheral surface.

2.3.4.3. Confirmation of cortical granules distribution patterns

Oocytes (N=100) stained with PNA were first categorized under wide-field fluorescence microscopy. Four different patterns of CGs were observed (Figures 2.2 and 2.3) as described previously (Yang et al., 2010; De los Reyes *et al*., 2011): 1) peripheral pattern where CGs were mainly in peripheral area characterized by a solid thin ring; 2) intermediate pattern where some of CGs were in the peripheral area as a thin ring shape; 3) cortical pattern where no thin ring within the peripheral area was observed; and 4) unclassified due to poor signal of PNA. These CG patterns were confirmed with confocal microscopy. The threshold point used to determine peripheral area against central area was 2 µm distance from the surface (peripheral area

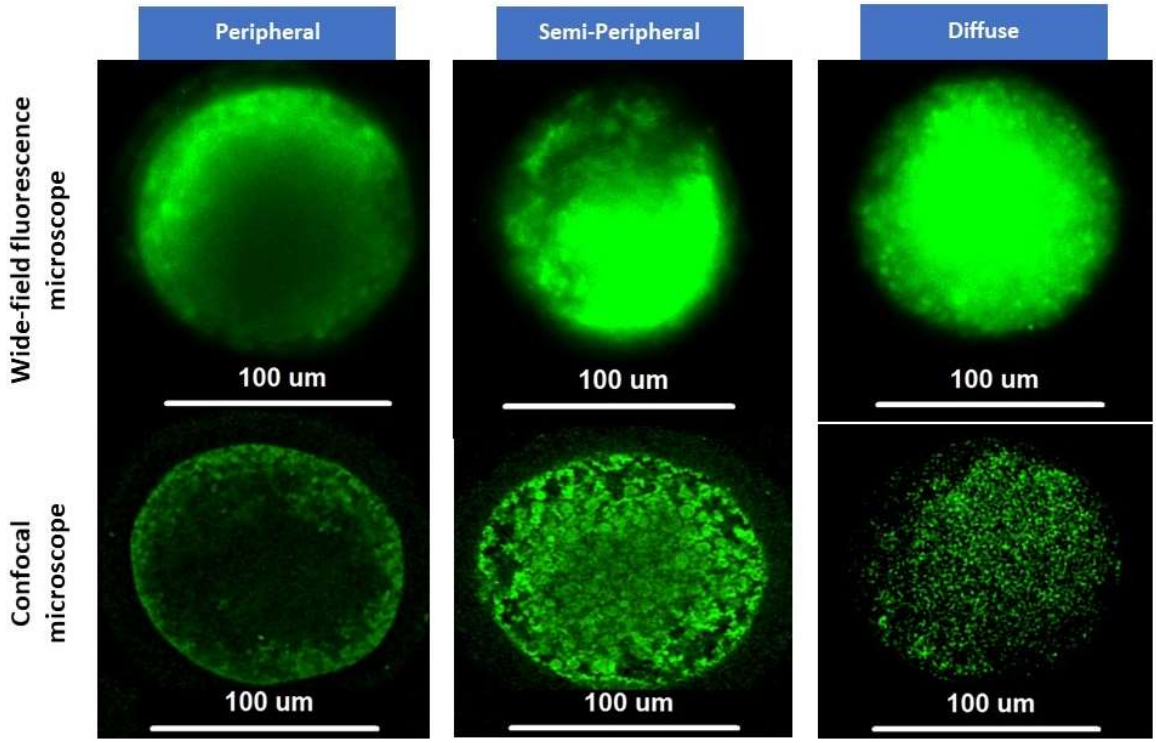

Figure 2.1: Three mitochondrial distribution patterns observed under wide-field fluorescence and confocal microscopes. Oocytes possessing diffuse pattern of mitochondria were considered "mature".

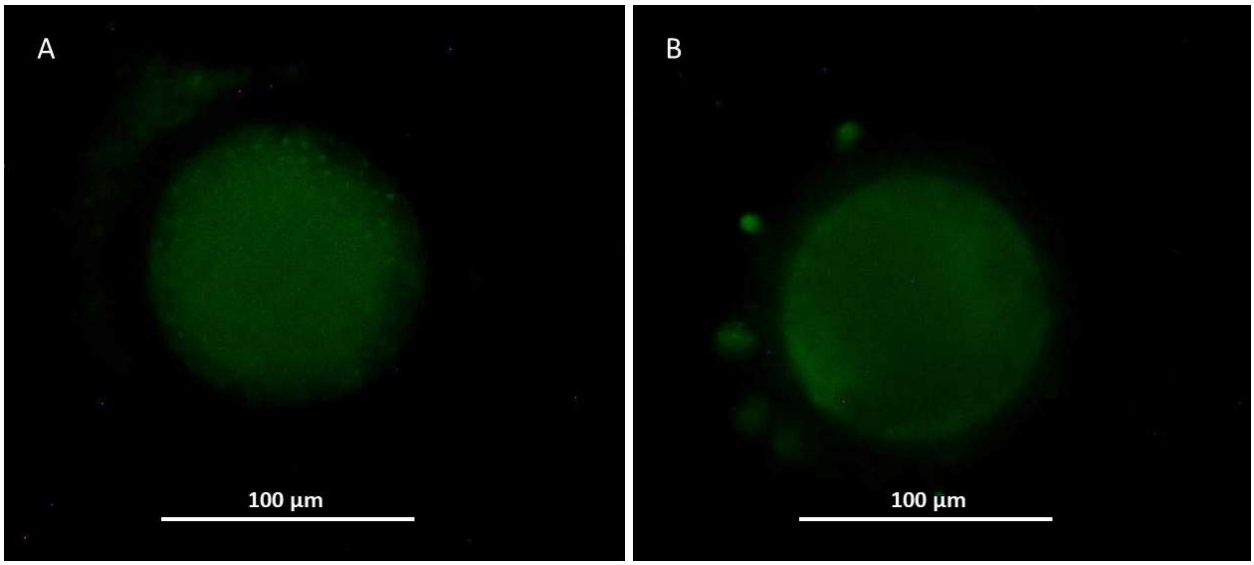

Figure 2.2: Unclassified patterns under the wide-field microscopy. The unclassified pattern of mitochondria (A) and unclassified pattern of cortical granules (B). The unclassified patterns in both mitochondria and cortical granules are due to the poor signal of dye.

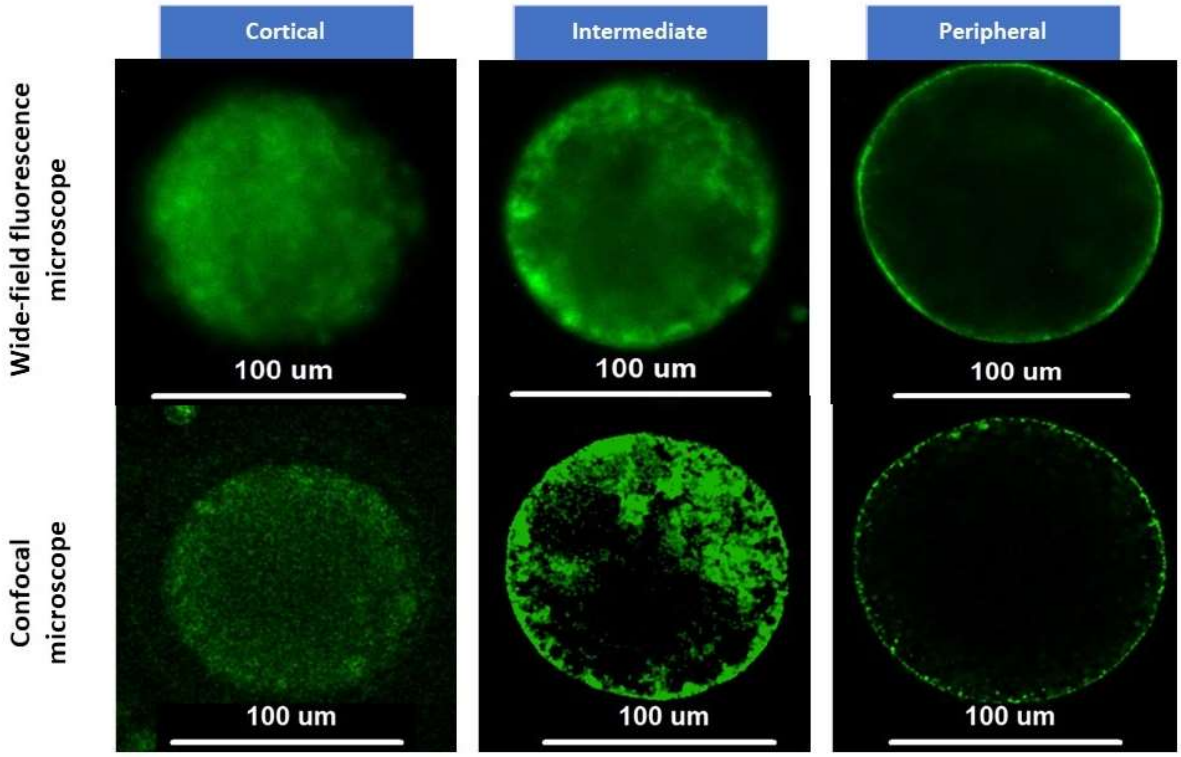

Figure 2.3: Three cortical granules distribution patterns observed under wide-field fluorescence and confocal microscopes. Oocytes possessing peripheral pattern of cortical granules were considered "mature".

2.4. Statistical Analysis

Data from 3 or more replicates was compiled and analyzed using STATA 17.0 software (Copyright 1985-2021 StataCorp LLC, 4905 Lakeway Drive, College Station, Texas 77845 USA). The percentage of oocytes within each repetition was the outcome variable used for all analyses. A zero-Skewness log transformation technique was applied before the statistical analysis to obtain a normal distribution. Differences across group were assessing using a one-way ANOVA was after the transformation. If significant, a pairwise comparison with Tukey's multiple group adjustment was used to confirm differences among groups. To compare the distribution of MT and CGs and the agreement between wide-field and confocal microscopy, kappa test was used. Data are expressed as means (%) ± standard deviation (SD). The minimum number of oocytes targeted for this study was 40, based on alpha of 0.05 and experiment power (beta) of 90%.

2.5. Results

2.5.1. Experiment 1: Oocyte Viability Using Fluorescent Dyes and Determination of Optimal Concentration.

Oocytes stained red due to PI uptake indicated dead oocytes while non-stained oocytes represented live oocytes (Figure 2.4). On the other hand, oocytes stained green due to FDA indicated live oocytes and the non-stained oocytes indicated dead oocytes (Figure 2.5). The viability rate of oocytes stained with WGA, RH-123, MTO, MTG, and PNA under fluorescent microscopy was determined to find the optimal concentration of these dyes (Figure 2.6). There was no significant effect in viability rate for concentrations of WGA tested. Viability was 49.3% ± 14.95 with the concentration of 1.3 µM in comparison with 55.0% ± 9.66 using 789 nM and 54.4% ± 14.99 using 526 nM concentrations. For RH-123, the viability rate was not significantly different: 87.3% ± 18.19, 88.7% ± 20.48 and 94.1% ± 10.70, at the concentrations tested (79 µM, 66 µM, and 26 µM, respectively). MTO had no significant effect on viability rate. The viability rate was 91.9% ± 1.02 for the concentration of 350 nM in comparison to 92.9% ± 2.93 at 200nM and 96.9% ± 2.86 at 100 nM concentrations, respectively. On the other hand, the viability rate using MTG differed significantly by concentration ($P \leq 0.05$), and was 36.7% ± 20.95 at 500 nM and 47.8% ± 13.49 at 450 nM concentrations in comparison with 75.4% ± 11.98 at 400 nM and 92.0% ± 5.21, 91.7% ± 4.46, 92.0% ± 6.93, and 94.7% ± 5.10 at the concentrations 350 nM, 300 nM, 250 nM, and 200 nM, respectively. The viability rate using PNA was 84.5% ± 7.32 at 833 nM concentration in comparison to 90.9% ± 0.83 at 625 nM concentration, and there was no significant difference between them. The control (non-stained oocytes) viability rate was 92.0% ± 5.45.

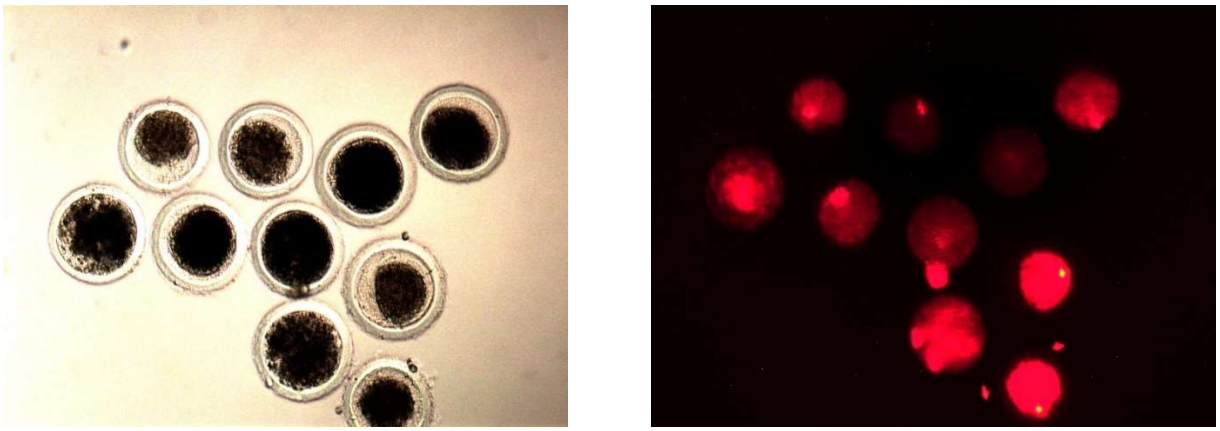

Figure 2.4: Pig oocytes matured *in vitro* under phase contrast microscope (left panel) and wide-field fluorescence microscope (right panel). In right panel, oocytes stained red due to uptake of propidium iodide are dead and oocytes which remained unstained are live.

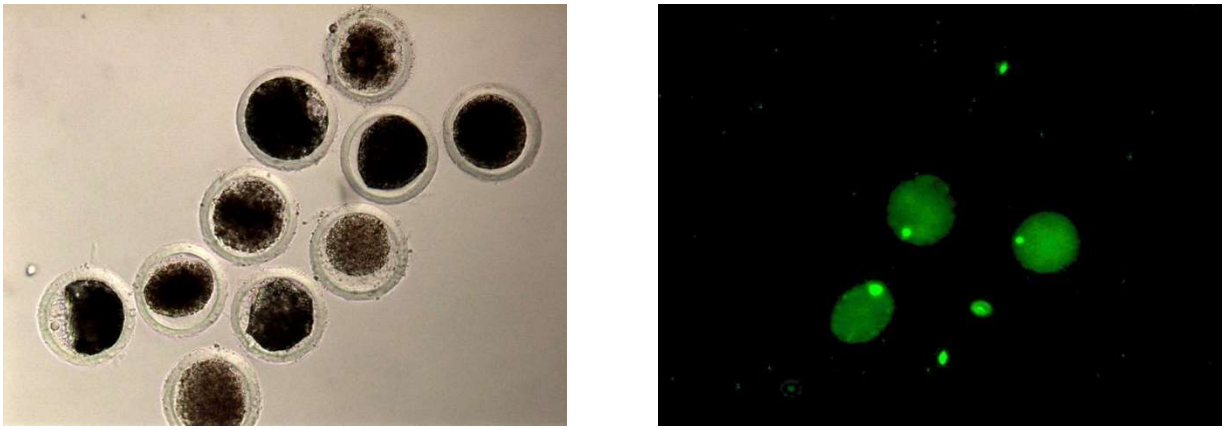

Figure 2.5: Pig oocytes matured *in vitro* under phase contrast microscope (left panel) and wide-field fluorescence microscope (right panel). In right panel, oocytes stained green due to uptake of fluorescein diacetate are live and oocytes which remained unstained are dead.

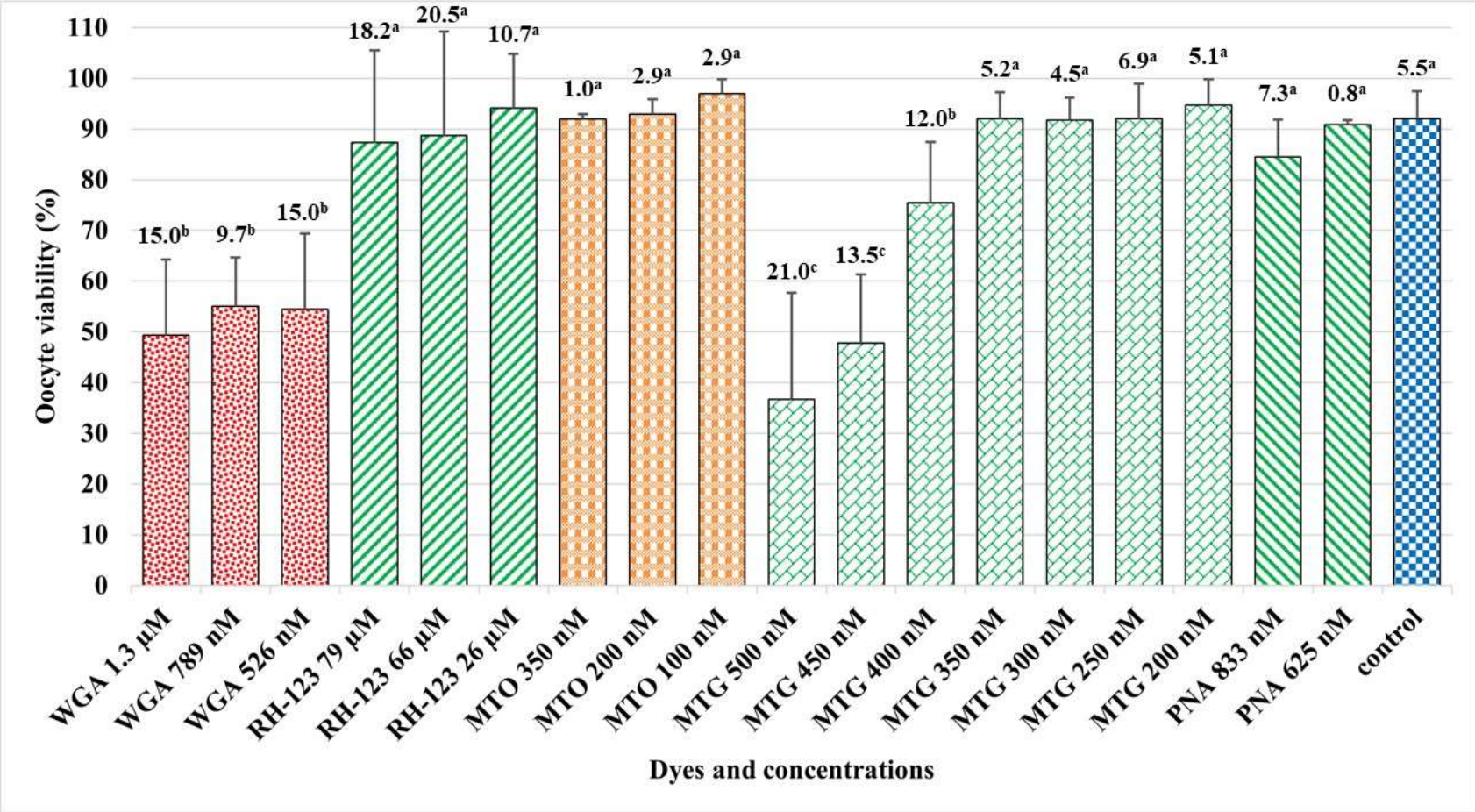

Figure 2.6: Effects of different concentrations of Wheat germ agglutinin (WGA), Rhodamine-123 (RH-123), MitoTracker orange (MTO), MitoTracker green (MTG), and Peanut agglutinin (PNA) on oocyte viability. Each bar represents the standard deviation (SD). Within a dye, SD with different letters (a-c) differ due to concentration ($P \leq 0.05$; number = 90-120 oocytes for each dye).

2.5.2. Experiment 2: Distribution of Mitochondria and Cortical Granules in Pig Oocytes

2.5.2.1. Confirmation of mitochondrial distribution

Based on confocal microscopy, the patterns of MT throughout the cytoplasm was assessed in Figure 2.1. These patterns (peripheral, semi-peripheral, and diffuse) were selected by the wide-field microscope firstly. This process was to confirm that we chose the correct pattern. The confirmation of the pattern was based on the statistics obtained from these oocytes depending on the mass of distribution of MT. In peripheral pattern, the majority of MT were immediately under the plasma membrane and fewer number of MT were present in the central area. In semi-peripheral pattern, an almost equal number of MT were present in the central area and beneath the plasma membrane while in the diffuse pattern, most of the MT (individual and clusters) were in the central area (Figure 2.7). In peripheral pattern group selected based on wide-field microscopy, 65% ± 6.5 (2493/3766) MT were in peripheral region of cytoplasm as compared with 35% ± 6.5 (1273/3766) of MT in central area. In semi-peripheral pattern group selected based on wide-field microscopy, 53% ± 3.2 (3205/6091) MT were in peripheral area of cytoplasm versus 47% ± 3.2 (2886/6091) MT in central region. In diffuse group selected based on wide-field microscopy, 41% ± 4.2 (3205/7825) of MT were in peripheral region of cytoplasm versus 59% ± 4.2 (4620/7825) of MT in central region (Table 2.1).

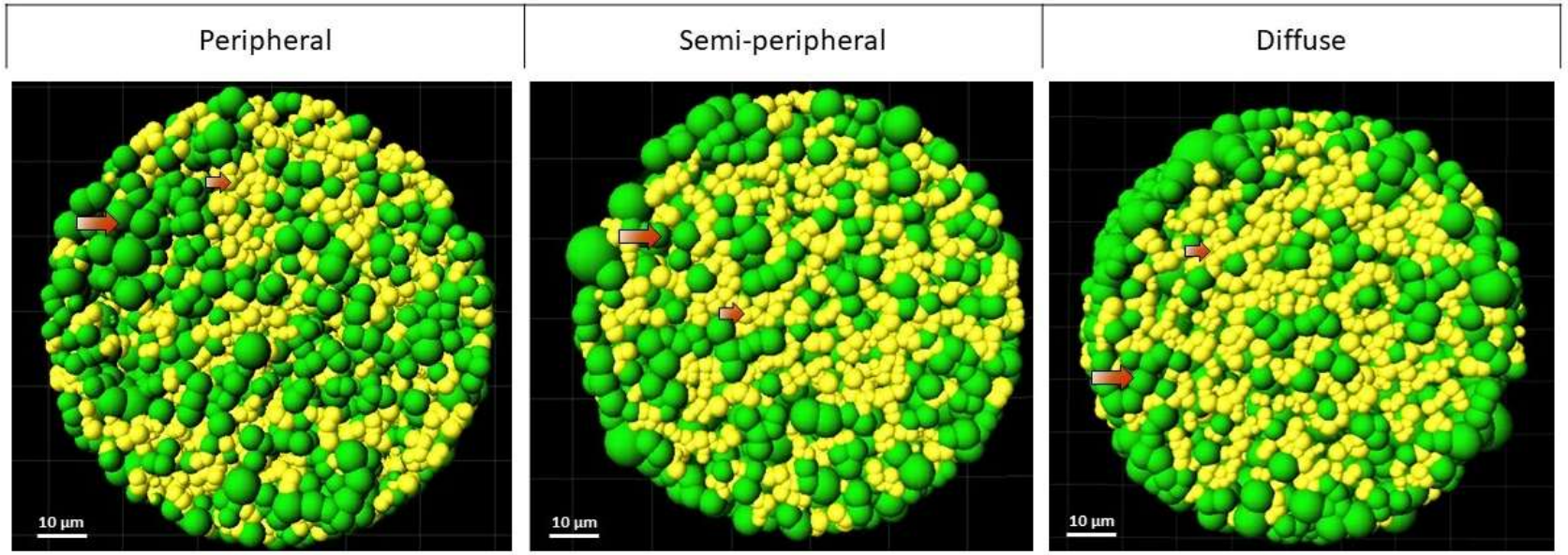

Figure 2.7: Mitochondrial distribution using Imaris software. The spaces between mitochondrial clusters and the mitochondria were measured with Imaris software. The green bubbles represent the spaces between the mitochondria (big arrows), and the yellow bubbles represent the mitochondria and the mitochondrial clusters (small arrows).

Table 2.1: Distribution of peripheral and central mitochondrial patterns within diffuse, semi-peripheral and peripheral patterns in *in vitro* matured pig oocytes after using Imaris (v.8) software. A total of 45 oocytes were used in three replicates (5 oocytes/group/replicate). Each value represents mean (%) ± SD.

Patterns selected by wide-field microscopy	Patterns measured by confocal microscopy	Mitochondria count
Peripheral pattern (n=15) *	Peripheral mitochondria	65 ± 6.5 [a] (N=2493) [64,64,68]
	Central mitochondria	35 ± 6.5 [b] (N=1273) [36,36,32]
Semi-peripheral pattern (n=15) NS	Peripheral mitochondria	53 ± 3.2 (N=3205) [54,53,52]
	Central mitochondria	47 ± 3.2 (N=2886) [46,47,48]
Diffuse pattern (n=15) *	Peripheral mitochondria	41 ± 4.2 [b] (N=3205) [38,43,41]
	Central mitochondria	59 ± 4.2 [a] (N=4620) [62,57,59]

* Within a specific pattern group (column), values with different letters (a and b) are different ($P \leq 0.05$) between the distribution of mitochondria.
NS represent non-significant difference in a column within a specific pattern group.
N represents number of mitochondria detected in confocal microscopy.
The numbers between square brackets represent the percentage of each replicate.

2.5.2.2. Confirmation of cortical granules distribution

Based on confocal microscopy, the patterns of CGs through the cytoplasm were assessed in Figure 2.3. These patterns (cortical, intermediate, and peripheral) were selected by the wide-field microscope firstly. This process was to confirm that we chose the correct pattern. Most of the CGs were in the peripheral area of the cytoplasm (underneath the plasma membrane) representing the peripheral pattern of CGs. In the cortical pattern, most of the CGs were in the central area of the cytoplasm and less number in the peripheral area of cytoplasm. In intermediate pattern, there was almost the same number of CGs in the central area or underneath the plasma membrane (Figure 2.8). In cortical pattern group selected based on wide-field microscopy, 25% ± 3.9 (3225/12793) of CGs were in peripheral region of cytoplasm versus 75% ± 3.9 (9568/12793) in central area. In intermediate pattern group selected based on wide-field microscopy, 49% ± 7.3 (2802/5570) of CGs were in peripheral area of cytoplasm versus 51% ± 7.3 (2768/5570) in central area. In peripheral pattern group selected based on wide-field microscopy, 71% ± 13.4 (1784/2491) of CGs were in peripheral area of cytoplasm versus 29% ± 13.4 (707/2491) in central region (Table 2.2).

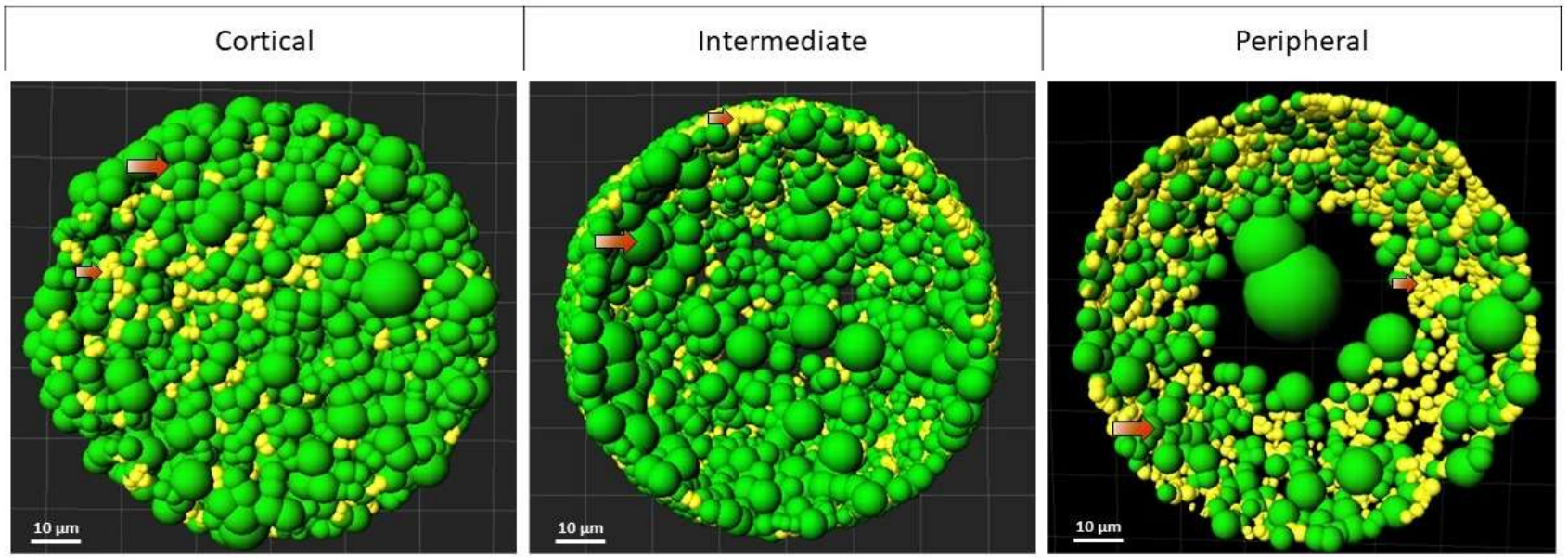

Figure 2.8: Cortical granules distribution using Imaris software. The spaces between cortical granules were measured with Imaris software. The green bubbles represent the spaces between the cortical granules (big arrows), and the yellow bubbles represent the cortical granules (small arrows).

Table 2.2: Distribution of peripheral and central cortical granules patterns within cortical, intermediate, and peripheral patterns in *in vitro* matured pig oocytes after using Imaris (v.8) software. A total of 45 oocytes were used in three replicates (5 oocytes/group/replicate). Each value represents mean (%) ± SD.

Patterns selected by wide-field microscopy	Patterns measured by confocal microscopy	Cortical granules (CGs) count
Cortical pattern (n=15) *	Peripheral CGs	25 ± 0.0 [b] (N=3225) [25,25,25]
	Central CGs	75 ± 0.0 [a] (N=9568) [75,75,75]
Intermediate pattern (n=15) NS	Peripheral CGs	49 ± 7.3 (N=2802) [48,45,53]
	Central CGs	51 ± 7.3 (N=2768) [52,55,47]
Peripheral pattern (n=15) *	Peripheral CGs	71 ± 13.4 [a] (N=1784) [66,75,72]
	Central CGs	29 ± 13.4 [b] (N=707) [34,25,28]

* Within a specific pattern group (column), values with different letters (a and b) are different (P≤0.05) between the distribution of cortical granules.
NS represent non-significant difference in a column within a specific pattern group.
N represents number of cortical granules detected in confocal microscopy.
The numbers between square brackets represent the percentage of each replicate.

2.5.2.3. Comparison between wide field microscope vs. confocal microscope in mitochondrial and cortical granules distributions.

Three patterns of MT distribution under wide-field fluorescence or confocal microscopes were observed (Figure 2.1): 1) peripheral pattern where the majority of MT were distributed in the peripheral area under the plasma membrane; 2) semi-peripheral where some MT were distributed in the central area and some in peripheral area; and 3) diffuse pattern where the majority of MT were distributed within the central area of cytoplasm. The peripheral and semi-peripheral patterns were considered as an indication of immature oocytes and the diffuse pattern as mature oocytes. There was 96.67% agreement between wide-field and confocal microscopy in the MT distribution. The expected agreement in this comparison was 56.67% with 0.92 ± 0.11 of Kappa test ($P \leq 0.01$; Table 2.3).

Similarly, three patterns of CGs distribution based on wide-field and confocal microscopes were observed (Figure 2.3): 1) cortical pattern – most of CGs distributed in the central area; 2) intermediate – some of CGs distributed in the central area and some beneath the plasma membrane as thin layer; and 3) peripheral pattern – most of the CGs distributed beneath the plasma membrane as a thin layer, like ring shape (Figure 2.3). The peripheral pattern of CGs was considered an indication of mature oocytes and the other patterns (cortical and intermediate) as immature oocytes. The agreement between wide-field and confocal microscopy using CGs was 97.78%. the expected agreement in this comparison was 55.56% with 0.95 ± 0.12 of Kappa test ($P \leq 0.01$; Table 2.4).

Table 2.3: Comparison between wide field and confocal microscope in mitochondrial distribution in *in vitro* matured oocytes derived from pre-pubertal gilts (n=45 oocytes in three replicates).

		Wide field		
	Pattern	Peripheral	Semi-peripheral	Diffuse
Confocal	Peripheral	15 (100%)	3 (20%)	0 (0%)
	Semi-peripheral	0 (0%)	12 (80%)	0 (0%)
	Diffuse	0 (0%)	0 (0%)	15 (100%)

The agreement was 96.67% ($P \leq 0.01$) with kappa value 0.92 ± 0.12. The expected agreement was 56.67%.

Table 2.4: Comparison between wide-field and confocal microscope cortical granules distribution in *in vitro* matured oocytes (n=45 oocytes in three replicates).

		Wide field		
	Pattern	Cortical	Intermediate	Peripheral
Confocal	Cortical	15 (100%)	1 (7%)	0 (0%)
	Intermediate	0 (0%)	14 (93%)	1 (7%)
	Peripheral	0 (0%)	0 (0%)	14 (93%)

The agreement was 97.78% ($P \leq 0.01$) with kappa value 0.95 ± 0.11. The expected agreement was 55.56%.

2.5.2.4. Mitochondrial and cortical granules distribution in pre-pubertal and adults sows

At the initiation of maturation, a significant majority of oocytes from pre-pubertal sows had peripheral and semi-peripheral patterns (37% ± 25.5 (51/139) and 30% ± 11.2 (41/139) respectively; Table 2.5) compared to a diffuse pattern ($P \leq 0.05$), suggesting that a majority of these oocytes were not matured. After IVM, the distribution shifted, and 48% ± 18.5 (64/135) of oocytes from pre-pubertal sows had a diffuse pattern (matured oocytes). This number of oocytes was significantly different from the peripheral pattern ($P \leq 0.05$), but it was not significantly different from semi-peripheral and unclassified patterns. Approximately 27% ± 7.0 (38/139) of the oocytes could not be classified at the initiation of the maturation; this number significantly dropped to ~19% ± 2.5 (26/135) at the end of the maturation period ($P \leq 0.05$; Table 2.5). Likewise, in pre-pubertal gilts the majority of adult sow oocytes showed peripheral and semi-peripheral patterns before maturation (Table 2.5). After IVM, 61% ± 10.2 (38/62) of sow oocytes had diffuse pattern, and it was significantly different compared to oocytes with a peripheral or a semi-peripheral pattern ($P \leq 0.05$). Interestingly, only a tiny proportion of oocytes from adult sows could not be classified in any pattern (Table 2.5).

Table 2.5: Mitochondrial distribution patterns in oocytes from pre-pubertal gilts and sow before and after 44-hr maturation using wide-field microscopy. Each value represents mean (%) ± SD.

Pattern	Pre-pubertal		Sow	
	Before maturation **	After maturation **	Before maturation **	After maturation **
Peripheral *	37 ± 25.5 a (x) (N=51) [12,63,37]	9 ± 3.5 b (x) (N=12) [12,9,5]	46 ± 22.0 a (x) (N=28) [61,57,21]	15 ± 2.5 b (x) (N=9) [12,14,17]
Semi-peripheral *	30 ± 11.2 a (x) (N=41) [38,17,34]	24 ± 12.7 b (y) (N=33) [38,19,14]	41 ± 4.4 a (x) (N=26) [39,38,46]	18 ± 2.0 b (x) (N=11) [15,19,17]
Diffuse *	6 ± 8.7 a (y) (N=9) [16,0,2]	48 ± 18.5 b (y) (N=64) [28,53,64]	11 ± 15.5 a (y) (N=8) [0,5,29]	61 ± 10.2 b (y) (N=38) [70,67,51]
Unclassified *”	27 ± 7.0 a (x) (N=38) [34,20,27]	19 ± 2.5 a (y) (N=26) [22,19,17]	2 ± 2.3 b (y) (N=1) [0,0,4]	6 ± 9.1 b (x) (N=4) [0,0,17]
Total oocytes	139	135	63	62

* Within a pattern of oocytes (row), values with different letters (a and b) represent differences (P≤0.05) between the pre-pubertal gilts and adult sows before and after maturation.
** within each maturation group (column), values with different letters (x and y) represent differences (P≤0.05) among patterns.
N represents number of embryos used.
*” Unclassified pattern was mainly due to cumulus cells surrounding oocytes or low fluorescent signal of MTG.
The numbers between square brackets represent the percentage of each replicate.

Before maturation, a significantly higher portion of oocytes from pre-pubertal gilts had an intermediate CGs pattern (48% ± 17.0; 64/135; $P<0.05$), compared to the other patterns (Table 2.6). At the end of the maturation period, different patterns of CGs were evenly distributed for oocytes from pre-pubertal gilts (ranging between 24% ± 5.1; 36/148; to 29% ± 7.4; 44/148). Interestingly, lesser number of oocytes had an intermediate pattern after maturation for 44 hrs compared to before maturation ($P\leq0.05$; Table 2.6). Also, proportion of oocytes with peripheral patterns after 44 hr maturation was greater ($P\leq0.05$) compared to before maturation. A low number of oocytes from pre-pubertal gilts could not be classified (Table 2.6) at 0 hr and 44 hrs.

Before maturation, sow oocytes had 52% ± 9.5 (31/61) of cortical pattern of CG, which was significantly different from intermediate and peripheral patterns ($P\leq0.05$; Table 2.6). After 44 hr maturation, a shift of distribution happened, and 69% ± 13.1 (42/62) of oocytes had a peripheral pattern that was significantly different from other patterns; $P\leq0.05$. In addition, oocytes matured for 44 hr had significantly less cortical and intermediate patterns compared to before maturation ($P\leq0.05$; Table 2.6). A small proportion of oocytes could not be classified at both before and after maturation stages.

Oocytes from adult sows had a significant proportion of oocytes with a cortical pattern at the beginning of maturation (52% ± 9.5; 31/61) compared to oocytes from pre-pubertal (16% ± 5.0; 10/62; $P\leq0.05$). A higher proportion of oocytes from pre-pubertal had an intermediate pattern (48% ± 17.0; 68/143) compare to oocytes from adult sows (16% ± 5.0; 10/62; $P\leq0.05$). According to peripheral pattern (mature oocytes), the oocytes from pre-pubertal gilts had higher proportion (19% ± 13.2; 27/143) than adult sows (15% ± 2.5; 9/61; $P\leq0.05$). After maturation, oocytes from pre-pubertal gilts had a significant portion of cortical pattern (24% ± 5.1; 36/148) compare to oocytes from adult sows (13% ± 3.8; 8/62; $P\leq0.05$). Similarly, the pre-pubertal gilts had a higher proportion of intermediate pattern after maturation (29% ± 7.4; 44/148) in comparison to adult sows oocytes (16% ± 5.0; 10/62; $P\leq0.05$). The oocytes from pre-pubertal gilts had a lesser proportion of mature oocytes (peripheral; 28% ± 9.5; 40/148) than oocytes from adult sows (69% ± 13.1; 42/62; $P\leq0.05$). Moreover, it was easier to classify oocytes from adult sows compared to oocytes from pre-pubertal pigs both before and after maturation (Table 2.6).

Table 2.6: Cortical granule distribution patterns in oocytes from pre-pubertal gilts and adult sows before and after maturation using wide-field microscopy. Each value represents mean (%) ± SD.

Pattern	Pre-pubertal		Sow	
	Before maturation **	After maturation **	Before maturation **	After maturation **
Cortical *	16 ± 4.6 a(x) (N=23) [20,17,11]	24 ± 5.1 b(x) (N=36) [30,23,20]	52 ± 9.5 c(x) (N=31) [59,55,41]	13 ± 3.8 a(x) (N=8) [11,10,17]
Intermediate *	48 ± 17.0 a(y) (N=68) [23,40,57]	29 ± 7.4 b(x) (N=44) [32,21,35]	25 ± 7.2 b(y) (N=15) [29,30,17]	16 ± 5.0 c(x) (N=10) [11,15,21]
Peripheral *	19 ± 13.2 a(x) (N=27) [14,34,9]	28 ± 9.5 b(x) (N=40) [20,38,24]	15 ± 2.5 c(y) (N=9) [12,15,17]	69 ± 13.1 d(y) (N=42) [78,75,54]
Unclassified *”	17 ± 7.4 a(x) (N=25) [20,9,23]	19 ± 1.7 a(y) (N=28) [18,18,21]	8 ± 14.4 b(z) (N=6) [0,0,25]	2 ± 4.6 b(z) (N=2) [0,0,8]
Total oocytes	143	148	61	62

* Within a pattern of oocytes (row), values with different letters (a and b) represent differences (P≤0.05) between the pre-pubertal gilts and adult sows before and after maturation.
** within each maturation group (column), values with different letters (x-z) represent differences (P≤0.05) among patterns.
N represents number of embryos used.
*” Unclassified pattern mainly due to cumulus cells surrounding oocytes or low fluorescent signal of PNA.
The numbers between square brackets represent the percentage of each replicate.

2.6. Discussion

As it is known, oocyte maturation has two steps to occur (nuclear and cytoplasmic maturation). In IVM, the nuclear maturation completed before the cytoplasmic maturation so there is lack of synchronization between nuclear and cytoplasmic maturation (SCHOEVERS et al., 2005). There is a main problem in IVM step due to lack of cytoplasmic maturation information and markers. Because the IVM step is the first and very important step in IVEP process. We tried to find cytoplasmic maturation markers (fluorescent dyes). There are many fluorescent dyes to detect cytoplasmic organelles, especially MT and CGs. There are several studies to evaluate these organelles through oocyte maturation using fixed oocytes. Our study is to evaluate some fluorescent dyes on the distribution of MT and CG organelles through oocyte maturation in unfixed oocytes to be first time reported. In addition, this is the first study to evaluate MT and CGs distribution using a comparison between the wide-field and confocal microscopy.

Oocytes used in this study were harvested from slaughtered pre-pubertal or adult sows and the estrous cycle stage of these pigs were not known. Therefore, it was assumed that harvested oocytes were at different maturation stages.

Since WGA yielded low viability of oocytes in all concentrations, it was considered as a harmful dye for the pig oocytes. Therefore, it was not used in subsequent experiments. The concentrations of WGA used in this study were according to previous studies on fixed mammalian oocytes (TAKAHASHI et al., 2013, NOVO et al., 2013). Although dye RH-123 demonstrated more than 85% viability for all concentrations and was found safe for pig oocytes. However, RH-123 has cumulative toxic effect on mouse blastocyst (THOUAS et al., 2004). For this reason, it was also omitted from subsequent experiments. Another dye, MTO, was also omitted from subsequent experiments due to lack of appropriate orange filter in our fluorescent microscope for evaluation. For all previous reasons, the MTG (200 nM) and PNA (625 nM) were selected for subsequent experiments. These concentrations had greater viability rates and were visualized clearly under wide-field microscope. These concentrations were considered as optimal concentrations to use.

Based on MT distribution under wide-field microscopy, the categories of oocytes were confirmed under confocal microscopy. In the peripheral MT pattern from oocytes segregated by wide-field microscopy, a sign of immature oocytes, 35% ± 6.5 (1273/3766) of MT were

distributed in the central area and 65% ± 6.5 (2493/3766) in the peripheral area. In the diffuse MT pattern selected by wide-field microscope, a sign of oocyte maturation, the distribution of MT after oocytes maturation was 59% ± 4.2 (4620/7825) in the central area vs. 41% ± 4.2 (3205/7825) in the peripheral area with confocal microscope. These data indicated that oocytes achieved sufficient cytoplasmic maturity during IVM process. In addition to central and peripheral MT patterns, there was a third pattern i.e. semi-peripheral pattern. In this pattern selected by wide-field microscope, 47% ± 3.2 (2886/6091) of MT were distributed in the central area vs. 53% ± 3.2 (3205/6091) in the peripheral area after measuring them by confocal microscope. These results proved that our technique of evaluation and sorting based on wide-field microscopy was successful in segregating different patterns of MT in pig oocytes.

Similar results were found on CG distribution in pig oocytes evaluated with wide-field and confocal microscopy. In cortical pattern based on wide-field microscopy segregating, the distribution of CGs using confocal microscope was 75% ± 3.9 (9568/12793) in central area vs. 25% ± 3.9 (3225/12793) in peripheral area in oocytes. These results indicate that these oocytes expressing a cortical pattern were still immature after the maturation process. However, in peripheral pattern based on wide-field microscopy selection, 71% ± 13.4 (1784/2491) of CGs were distributed in peripheral area vs. 29% ± 13.4 (707/2491) in the central area of oocytes and oocytes in this category were considered as mature. The third pattern was the intermediate distribution in which the distribution of CGs in central and peripheral areas were almost 50:50. These results on MT and CGs distributions proved that our technique of oocyte sorting based on wide-field microscopy was valid.

To compare the findings between the wide-field and confocal microscopes, there was 96.67% agreement of MT with expected agreement of 56.67% ($P \leq 0.01$) and Kappa test 0.92 ± 0.12. These results indicated the segregation of oocytes based on CGs distribution using the wide-field microscope was accurate and almost perfect (LANDIS and KOCH, 1977). On the other hand, the 20% error between the semi-peripheral and peripheral patterns occurred due to poor signal of MTG dye or the nuclear position within the cytoplasm. The agreement of CGs distribution was 97.78% with expected agreement 55.56% ($P \leq 0.01$) and 0.95 ± 0.12 Kappa test. This agreement indicated to almost perfect accuracy (LANDIS and KOCH, 1977). The difference of 7% likely due to the poor signal of the PNA dye. Another reason for this error was the similarity between peripheral pattern and intermediate pattern. Both patterns have a thin layer

of CGs in the cytoplasm, but in the peripheral pattern there was a completely thin ring shape in contrast to an incomplete ring in the intermediate pattern (YANG et al., 2010).

Using MTG, our study demonstrated that 37 ± 25.5 (51/139) to 46% ± 22.0 (28/63) of the oocytes from both pre-pubertal and adult sows did not initiate a translocation of MT from peripheral to diffuse patterns. So, the other portion of oocytes had already initiated or completed the translocation of MT to reach an organization associated with oocyte maturation (SUN et al., 2001). After 44 hr of maturation, a significant portion of oocytes demonstrated translocation of MT to a diffuse distribution associated with mature oocytes i.e. 48% ± 18.5 (64/135) for pre-pubertal and 61% ± 10.2 (38/62) for sow. We did not observe a statistical difference in the MT distribution between age groups ($P \leq 0.05$), which is in agreement with a previous study (PAWLAK et al., 2016b). Thus, we can assume that pre-pubertal and sow oocytes likely an equal potential to translocate MT to a diffuse pattern after 44 hr of incubation.

CGs distributions at the time of collection showed that a small proportion of oocytes from pre-pubertal gilts had a cortical pattern (16% ± 4.6; 23/143); thus, 84% of oocytes had already initiated maturation or already matured. The majority of oocytes from adult sows (52% ± 9.5; 31/61) had cortical pattern at the initiation of the maturation period. Unfortunately, this early initiation of the CGs translocation in oocytes from pre-pubertal gilts produced a smaller proportion of oocytes with peripheral pattern after 44 hr of maturation (28% ± 9.5; 40/148), compared to sow oocytes (69% ± 13.1; 42/62; Table 2.6). These results suggest that adult sow oocytes were fully competent to translocate CGs before and after maturation, which is partially present on pre-pubertal oocytes (MARCHAL et al., 2001). Translocation of CGs is accomplished via the actin filament (LIU, 2011, CHEESEMAN et al., 2016a), while MT are translocated using microtubules (YAMOCHI et al., 2015). We can speculate that a mechanism related to the control of actin polymerization is not optimal for the translocation of CGs of oocytes in pre-pubertal animals (VELILLA et al., 2005).

Unclassified oocytes in both MT and CGs were due to the poor signal of the fluorescent dyes. Thus, in before maturation groups, this unclassified pattern was due to the cumulus cells surrounded the oocytes. These cells prevent the clear visibility of the cytoplasm of oocytes.

2.7. Conclusions

In summary, four out of five dyes used in this experiment were safe for pig oocytes. A wide-field microscope was found to be comparable to a confocal microscope (96.67% and 97.78% agreements) in sorting of oocyte based on cytoplasmic maturation. The wide-field microscope is easier and faster in sorting oocytes based on MT and CGs distributions. There were three main patterns for the MT distribution: peripheral, semi-peripheral, and diffuse. The peripheral and semi-peripheral patterns indicated to immature or maturing oocytes while the diffuse pattern was assigned to mature oocytes. Also, there were three main patterns for CG distribution: cortical, intermediate, and peripheral. The cortical and intermediate patterns were assigned to immature or maturing oocytes, and the peripheral pattern assumed to be mature oocytes. Our recommendation is to use wide-field (inverted) fluorescence microscope for an easy and fast method to segregate pig oocytes through MT and CGs distribution by staining oocytes with 200 nM of MTG and 625 nM of PNA. We recommend also to use sow oocytes in IVEP procedure due to full competency of maturation.

CHAPTER 3: PRE-SELECTION OF MATURED OOCYTES IMPROVES THE QUALITY OF *IN VITRO* PRODUCED PIG EMBRYOS

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Relationship of this study to the dissertation

We evaluated the mitochondrial (MT) and cortical granules (CGs) distribution before and after maturation in previous chapter (chapter 2). In the current chapter, we evaluated the ability of stained oocytes to undergo *in vitro* fertilization (IVF) and subsequently develop into embryos. In this chapter, we evaluated the cleavage, morula, and blastocysts rates in oocytes sorted based on the mitochondrial and cortical granules distribution. We hypothesized that sorted oocytes with appropriate distributions of MT or CGs in mature oocytes would fertilize, and a significant portion of the presumptive embryos will develop to the blastocyst stage compared to the non-sorted oocytes.

Authors' Contributions

Hamza Al-Maamory: Performed the study, experimental design, write the manuscript.
Carl Lessard: Supervised and participated in the experimental design and critical revision the manuscript.
John Harding: Critical notes to revise the manuscript and strategies for statistical analysis.
Crissandra Auckland: Collaboration of IVEP procedures

3.1. Abstract

The low efficiency of *in vitro* production of embryos (IVEP) in pigs could be due to the insufficient cytoplasmic maturation. Mitochondrial (MT) and cortical granules (CGs) distribution and patterns are connected to the status of the cytoplasmic maturation of oocytes. Thus, this study hypothesized that sorted oocytes having an appropriate distribution or location of MT or CGs in mature oocytes would fertilize, and a significant portion of the presumptive embryos will develop to the blastocyst stage compared to non-sorted oocytes. The objectives of this study were to evaluate: 1) ability of sorted oocytes to sustain embryo development after *in vitro* fertilization; and 2) polyspermy rates in relation to CG distribution using the pronuclear test. Grade-1 and -2 oocytes were harvested from pre-pubertal gilts or sows. Oocytes were matured *in vitro*, cumulus cells were denuded, and stained with MitoTracker Green (MTG) to evaluate the MT distribution and Peanut Agglutinin coupled with FITC (PNA) for CGs distribution. The stained oocytes were grouped based on three distribution patterns observed for MT and CGs and were separately fertilized following standard *in vitro* fertilization (IVF) procedure. Cleavage, morula, and blastocyst rates were evaluated. The quality of 7-day old blastocysts was also assessed according to the International Embryo Transfer Society guidelines. Results revealed that prepubertal and sow oocytes sorted based on the location of MT or CGs can be fertilized and develop into embryos, similar to the non-stained oocytes. The cleavage, morula and blastocyst rates ranged from 65 to 75%, 25 to 45% and 7 to 15%, respectively. Interestingly, sorted groups demonstrated lower polyspermy and a higher monospermic fertilization rate ($P \leq 0.05$) compared to non-sorted group (12% ± 1.53; 11/89 versus 28% ± 3.06; 18/65 and 64% ± 5.20; 57/89 versus 50% ± 1.00; 90/181 respectively). A higher quality of blastocysts was produced in the sorted oocyte group than non-sorted oocytes used for standard IVF. There was a significant decline in polyspermy of the oocytes with peripheral pattern in comparison to other groups ($P \leq 0.05$). In summary, this study demonstrated that oocytes can be stained, sorted and fertilized having potential to develop into embryos. The blastocyst rate did not differ between sorted and not sorted oocytes, but the quality of the blastocysts from sorted oocytes was higher compared to non-sorted oocytes. In addition, the sorting method based on CGs distribution can decrease polyspermy fertilization.

3.2. Introduction

Success in *in vitro* production (IVEP) of embryos relies on the acquisition of oocyte competence during *in vitro* maturation process (IVM), during which the oocyte completes its nuclear and cytoplasmic maturations. In general, IVM culture conditions that are currently used for porcine oocytes yield high rates of nuclear maturation (80-90%) (KĄTSKA-KSIĄŻKIEWICZ, 2006). However, blastocyst rates for these matured porcine oocytes would reach only 30% under the best culture conditions (FOWLER et al., 2018) indicating that cytoplasmic maturation of oocytes may not be completed during the IVM procedure. This notion is further supported by observation that *in vitro* matured oocytes are usually less competent to develop to the blastocyst stage than *in vivo* matured oocytes (NAKAMURA et al., 2017). Oocytes that are matured *in vivo* undergo changes in organelle number and distribution pattern during the growth of follicles that take days to complete (DADARWAL et al., 2015, READER et al., 2017). Evidently, a heterogenous population (duration of follicular growth) and comparatively short duration of IVM results in oocytes with incomplete cytoplasmic maturation, negatively affecting their *in vitro* developmental competency.

Reorganization of both mitochondria (SUN QY et al., 2001) and cortical granules (LIU, 2011) within the ooplasm has been used in past to evaluate the cytoplasmic maturation of porcine oocytes. Mitochondria (MT) play a crucial role in oocyte maturation as they provide energy and regulate calcium homeostasis as well as degradation pathways (RAMALHO-SANTOS et al., 2009, BABAYEV and SELI, 2015). MT translocate from the peripheral area of oolemma to a homogenous distribution inside the cytoplasm in porcine oocytes undergoing IVM (BREVINI et al., 2005, SUN QY et al., 2001). Improper IVM conditions such as removal of porcine follicular fluid result in MT maintaining a peripheral distribution in 57% of porcine oocytes (BREVINI et al., 2005). Cortical granules (CGs) are secretory organelles that release proteinases responsible for modifying the zona pellucida to prevent polyspermy (LIU, 2011, READER et al., 2017). At completion of cytoplasmic maturation, CGs relocate from the peripheral region to lie adjacently as a small layer under the oolemma of the oocyte (PAWLAK et al., 2012). Interestingly, redistribution of CGs is delayed for oocytes of pre-pubertal gilt compared to sow oocytes (PEDERSEN et al., 2016).

Distribution patterns of MT and CG within the porcine oocytes have been studied in past using MitoTracker (MTG) and Peanut-Agglutinin (PNA) coupled with a fluorescent-isothiocyanate (FITC) dyes, respectively (LIU, 2011, SUN QY et al., 2001, BREVINI et al., 2005, YOSHIDA et al., 1993). Further, PNA-FITC did not interfere in the fertilization process, and the dye gradually faded away at 18hrs post-fertilization (YOSHIDA et al., 1993). The objectives of our study were to use MTG and PNA-FITC to identify immature versus mature porcine oocytes and to compare the sorted oocyte groups for their fertilization and blastocyst rates. Our hypothesis was that sorted oocytes, having a location of MT or CGs associated with "matured" oocytes, will be fertilized and a significant portion of the presumptive embryos will develop to the blastocyst stage compared to the non-sorted oocytes. Our results demonstrated that "matured" sorted oocytes can be fertilized and sustain growth of embryos to expanded blastocysts. However, the number of blastocysts produced did not improve compared to the standard IVF procedures.

3.3. Materials and Methods

3.3.1. Animals and Chemicals

Oocytes used in this experiment were derived from pre-pubertal gilts and adult sows slaughtered from local abattoirs. Ovaries from adult sows and pre-pubertal gilts were obtained from Valley Meat Processors (40 km north of Saskatoon) and Country Choice Meats (81 km north of Saskatoon), respectively. All chemicals were purchased from the Sigma Chemical Co, Oakville, Canada, unless otherwise mentioned below. All media were filtered with a 0.22 μm PVDF filter (Argos technology, Elgin, USA) for this study.

3.3.2. Oocytes Collection and *In Vitro* Maturation

Ovaries were placed into an insulated container (35-37 ºC) during transport to Westgen Lab, University of Saskatchewan. After removal of surrounding non-ovarian connective tissues, ovaries were washed (3X) in sterile saline (0.9% NaCl, at 22 °C) and immersed in glucose

solution (0.128 M, at 22 °C) for 30 min. Following this, cumulus–oocyte-complexes (COCs) were harvested from three to eight-mm (diameter) follicles, and only Grade I and II (COCs) (MARCHAL et al., 2002) were retained for further processing. These oocytes were washed (3X) with washing medium (TCM-199, Invitrogen, Burlington, Canada; supplemented with 25 mM of HEPES and 50 mg/ml gentamycin; maintained at 37 ˚C). The selected COCs were rinsed (1X) in maturation medium (TCM-199, 3.05 mM D-glucose, 0.1% polyvinyl alcohol, 0.91 mM sodium pyruvate, 50 mg/ml gentamycin supplemented with 0.57 mM L-cysteine, 10 ng/ml epidermal growth factor (EPG), 0.01 U/ml Follitropin (Bioniche, Oakville, Canada) and 5 µg/ ml Luteinizing Hormone (Lutropin-V®; Bioniche, Belleville, Canada). Subsequently, COCs were transferred into a 4-well dish (maximum 50 COCs/well containing 500 µl of maturation medium) and incubated at 38.5˚C 5% CO_2, 5% O_2 and 90% N_2 and high humidity, for 44 hrs under a controlled environment.

3.3.3. Staining of Oocytes

Before maturation, a portion of oocytes were denuded using 0.1% hyaluronidase in TCM-199 (Invitrogen, Burlington, Canada) with a series of pipetting (approximately 80 times) using 200 ul pipette set at 100µl. Another portion of oocytes were denuded after 44 hrs of maturation using the same procedure. Denuded oocytes were washed twice with phosphate buffered saline (PBS; ThermoFisher Scintific, Gibco, Canada) with 0.1 % polyvinyl alcohol (PVA) and then transferred into 50 µl droplets of 0.1% PVA-PBS buffer with 200 nM of MitoTracker Green (MTG, Invitrogen, Burlington, Canada) or with 625 nM of PNA, determined in chapter 2, for 30 min. After incubation, the stained oocytes were washed (3X) with 0.1% PVA-PBS buffer and transferred into the same medium. A wide-field stereo epi-fluorescence microscope (Olympus SZX16, Japan) was used to visualize MT and CGs locations (Zoom: 80X, Excitation: 460-490, and Emission: 510 longpass). Oocytes stained with MTG were sorted, according to MT distribution, into four groups: peripheral, semi-peripheral, diffused and un-classified categories (Figure 2.10 chapter 2) based on previous studies (SUN QY et al., 2001, DE LOS REYES et al., 2011, BREVINI et al., 2005). Oocytes (n=100) stained with PNA were sorted, according to CGs distribution, into four groups: cortical, intermediate, peripheral and un-classified categories (Figure 2.11 chapter 2) based on previous study (YOSHIDA et al., 1993)).

3.3.4. Oocytes Fertilization and Semen

In vitro fertilization (IVF) was used to assess the ability of sorted oocytes to assess the embryo development. After IVM, oocytes were denuded as described in Section 3.3.3. A portion of the denuded oocytes was stained and sorted as previously described. The other portion was used for controls without staining. Oocytes designated as control were washed (3X) in a modified Tris-buffered medium (mTBM supplemented with 0.2% bovine serum albumin (BSA; Minitube, Verona, USA) and 1 mM caffeine). The washed and/or stained oocytes were transferred into mTBM that was used as a fertilization medium. Frozen semen from elite boar with proven fertility was used for *in vitro* fertilization. Straws were thawed at 37 °C for 20 seconds and diluted with mTBM media and centrifuged at 1000 × g for 5 min twice and resuspended in 1ml of mTBM. Motility and concentration of sperm cells were analyzed using a Computer Assisted Sperm Analyzer (Spermvision, Minitube Canada). A total of 50,000 motile sperm cells were incubated with matured oocytes at 38.5°C in a controlled environment (5% CO_2, 5% O_2 and 90% N_2) for 6 hr.

3.3.5. Embryo Culture

Presumptive zygotes were washed (3X) with a modified NCSU23 with glucose replaced by sodium lactate (4.5 mM) and sodium pyruvate (0.33 mM), then cultured in this media supplemented with 0.4% BSA at 38.5°C in an environment of 5% CO_2, 5% O_2 and 90% N_2 for 48 hrs. After culture, embryos were transferred into a non-modified NCSU23 (with 5.55 mM of glucose) for additional 7 days. Cleavage rate and embryo development were evaluated on days 3, 5, and 7 after fertilization. The quality of blastocysts produced (day 9 post-fertilization) was subjectively evaluated using the criteria established by the International Embryology Transfer Society (BÓ and MAPLETOFT, 2013). According to this evaluation, the quality code 1 (Excellent or good quality) indicates to the individual blastomeres are homogeneous in size, color, and density to form a balanced and spherical mass in the embryos. This embryo is developing at the normal stage. Abnormalities should be minimal, and at least 85% of the molecules should be a surviving, complete embryonic mass. The quality code 2 (Fair quality) indicates the general form of the embryonic mass, as well as the size, color, and density of single

cells, are slightly irregular in these embryos. At minimum 50% of the embryonic weight must be undamaged. In quality code 3 (Poor quality), these embryos demonstrate significant abnormalities in the form of the embryonic mass as well as in the size, color, and density of single cells. At least 25% of the embryo mass must be undamaged.

3.3.6. Experiment 1: Evaluation of *In Vitro* Embryos Production from Sorted Oocytes According to Distribution of Mitochondrial or Cortical Granules

3.3.6.1. *In vitro* production and quality of pig embryos using sorted oocytes from pre-pubertal gilts

In vitro matured oocytes from pre-pubertal gilts were divided into 4 main groups (Table 3.1): Group 1 (standard IVF), oocytes were not stained but were processed for IVF (positive control, n=113 for MT and n=153 for CGs); Group 2 (Stained & no IVF), oocytes were stained but not processed for IVF procedure (negative control, n=97 for MT and n=89 for CGs); Group 3 (No stain & no IVF), oocytes were not stained (for MT or CGs) and not processed for IVF (negative control; n=96 for MT and n= 90 for CGs); Group 4, oocytes were stained, categorized (for CG or MT distribution patterns) and processed for IVF procedure (sorted group, n=298 for MT and n=302 for CGs) This group was subcategorized based on MT and CGs patterns.

Table 3.1: Number of oocytes and replicates used in the *in vitro* production of embryos

Mitochondrial distribution					
Group		# of oocytes			
		Pre-pubertal (3 replicates)		Sow (5 replicates)	
Standard IVF		113		85	
Stained & no IVF		97		-	
No stain & no IVF		96		84	
Sorted oocytes	Peripheral	58	Total 298 oocytes	31	Total 178 oocytes
	Semi-peripheral	67		37	
	Diffused	135		107	
	Unclassified	38		3	
Cortical granules distribution					
Group		# of oocytes			
		Pre-pubertal (3 replicates)		Sow (4 replicates)	
Standard IVF		153		87	
Stained & no IVF		89		-	
No stain & no IVF		90		84	
Sorted oocytes	Cortical	51	Total 302 oocytes	35	Total 198 oocytes
	Intermediate	65		36	
	Peripheral	142		120	
	Unclassified	44		7	

3.3.6.2. *In vitro* production and quality of pig embryos using sorted oocytes from adult sows

Due to lower number of COCs obtained from sows, *in vitro* mature oocytes were distributed into 3 main Groups (Table 3.1): first group was standard IVF, oocytes were not stained but were processed for IVF (positive control, n=85 for MT and n=87 for CGs). The second group was no stain & no IVF group, these oocytes did not stain (MT or CGs) and did not process for IVF (negative control, n=84 for MT and 84 for CGs), and third group of oocytes were stained, categorized (for CGs or mitochondria distribution patterns) and processed for IVF procedure (sorted group, n=178 for MT and n=198 for CGs). This group also subcategorized based on MT and CGs distribution.

3.3.7. Experiment 2: Evaluation of Polyspermy in Pig Embryos from Sorted Oocytes Based on Cortical Granules Distribution

After 6 hr of fertilization, pre-pubertal gilts' oocytes from Groups 1 (Standard IVF) and 4 (stained for CGs) were washed (3X) with a washing solution (PBS + 0.1% PVA). Presumptive fertilized oocytes were transferred into trypsin droplet (0.25% trypsin with 0.02% EDTA) for 5 min to remove the accessory sperms around oocytes. After 5 min, fertilized oocytes were transferred in to blocking media (PBS + 2% BSA) and then washed (2X) in blocking media (5 min each). Trypsin-treated oocytes were fixed using 4% paraformaldehyde for overnight. The next day, oocytes were washed (2X) in washing solution and then transferred into a droplet (5 µl) of Vectashield with 4',6-diamidino-2-phenylindole (DAPI). Number of pronuclei were observed using a fluorescent microscope (Carl Zeiss, Axioskop 40, Germany; 40X) to evaluate polyspermy.

3.4. Statistical Analysis

Data from 3 or more replicates was compiled and analyzed using STATA 17.0 software (Copyright 1985-2021 StataCorp LLC, 4905 Lakeway Drive, College Station, Texas 77845 USA). The percentage of oocytes or embryos within each repetition was the outcome variable used for all analyses. A zero-Skewness log transformation technique was applied before the

statistical analysis to obtain a normal distribution. Differences across group were assessing using a one-way ANOVA was after the transformation. If significant, a pairwise comparison with Tukey's multiple group adjustment was used to confirm differences among groups. The differences between groups in IVEP development experiments were based on their means. Data were expressed as mean (%) ± standard deviation (SD). For the assessment of embryos quality, Mann-Whitney test was used, and data were expressed as the percentage of embryos ± SD.

3.5. Results

3.5.1. Experiment 1: Evaluation of *In Vitro* Embryo Production from Sorted Oocytes According to Distribution of Mitochondrial or Cortical Granules

3.5.1.1. *In vitro* production and quality of pig embryos using sorted oocytes of pre-pubertal gilts

Sorting of oocytes based on mitochondrial distribution and embryo development:

Embryos (day 3) derived from standard IVF (75% ± 1.00; 85/113) and diffused MT patterns (77% ± 1.00; 104/135) had significantly higher cleavage rate in comparison to other groups (P≤0.05; Figure 3.1). Cleavage rate of peripheral (45% ± 5.00; 26/58), semi-peripheral (55% ± 5.00; 37/67), and unclassified (60% ± 4.04; 24/38) groups were significantly higher (P≤0.05) than the negative controls (stained & no IVF; 17% ± 5.51; 19/97, and no stain & no IVF; 24% ± 6.51; 24/96). At the morula stage, the standard IVF and diffused MT groups had approximately 30% of morula (29% ± 0.58; 32/113, and 33% ± 3.15; 44/135, respectively), which was significantly higher than the other groups (P≤0.05; Figure 3.1). The semi-peripheral (14% ± 1.53; 9/67) and unclassified (10% ± 11.02; 5/38) groups had significantly higher morula rates than the negative controls (P≤0.05). Only the peripheral group (6% ± 2.65; 4/58) did not differ from the negative controls at the morula stage (P>0.05). At the blastocyst stage, standard IVF (9% ± 1.53; 10/113) and diffused (13% ± 0.58; 17/135) groups had higher development rate (day 7) compared to the other groups (P≤0.05). Gross quality comparison revealed that the diffused group had more (P≤0.05) early blastocysts and blastocyst stage with quality code 1, but

not for expanded blastocysts (100% ± 0.00 and 78% ± 38.70, respectively) compared to the standard IVF group (0% ± 0.00 and 8% ± 14.40, respectively; Figure 3.2 and Figure 3.3).

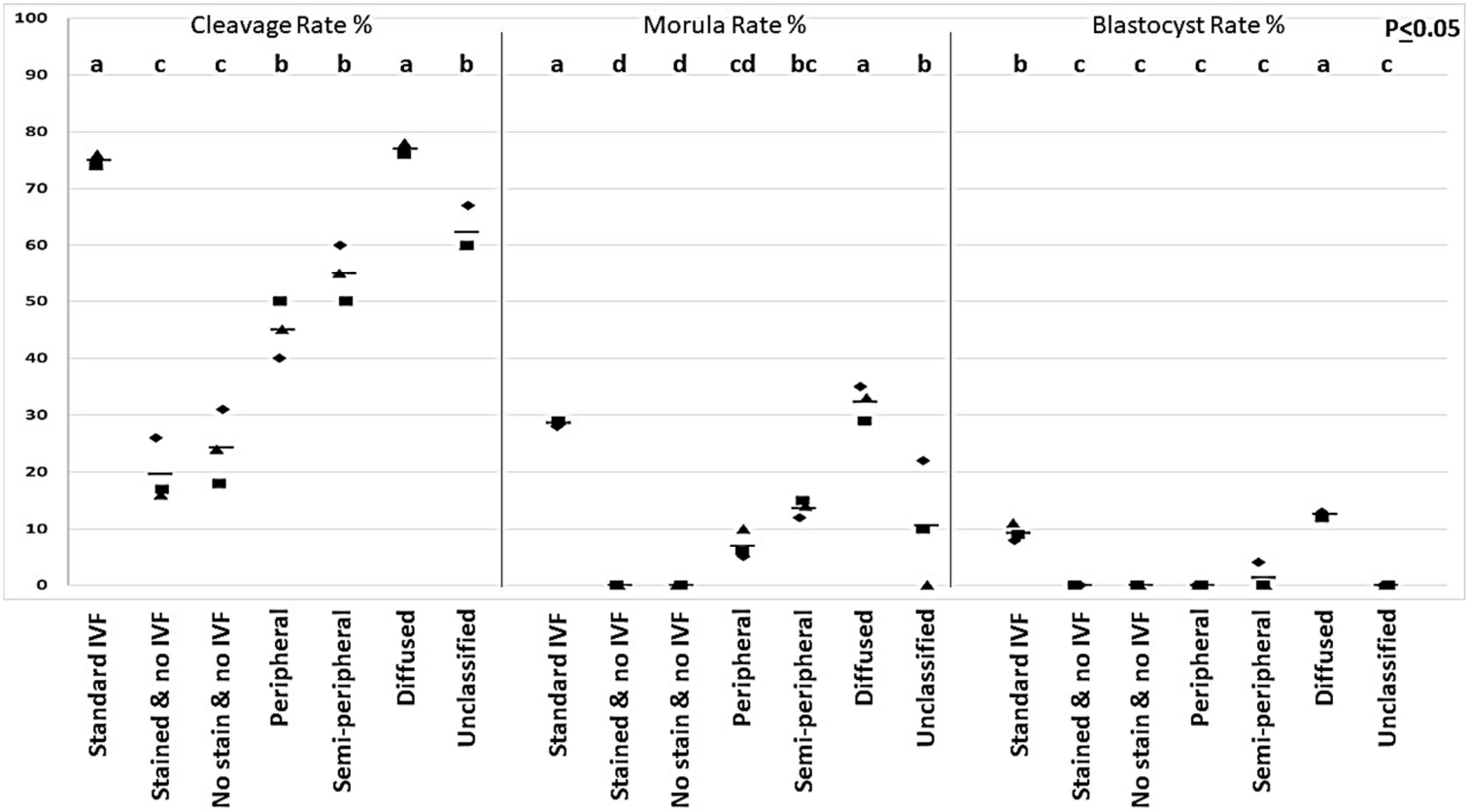


Figure 3.1: Fertilizing ability and embryo development of pre-pubertal gilts oocytes, sorted on the basis of distribution of mitochondria. Each group was evaluated for the cleavage (day 3), morula (day 5), and blastocyst (day 7) rates. Oocytes stained with MTG were classified as: peripheral, semi-peripheral, diffused, and unclassified. Control groups were standard IVF without staining), oocytes stained but no IVF (stained & no IVF), and no stain & no IVF. Different letters within the same embryo development stage indicates a significant difference ($P \leq 0.05$). The mean of the average rate was represented by the horizontal bar for each group.

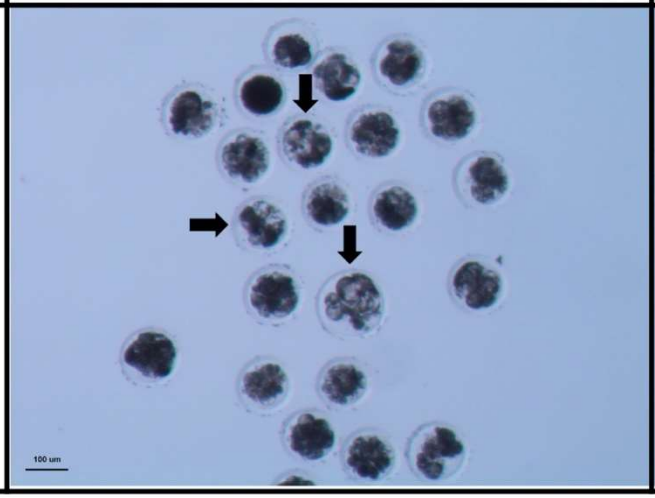
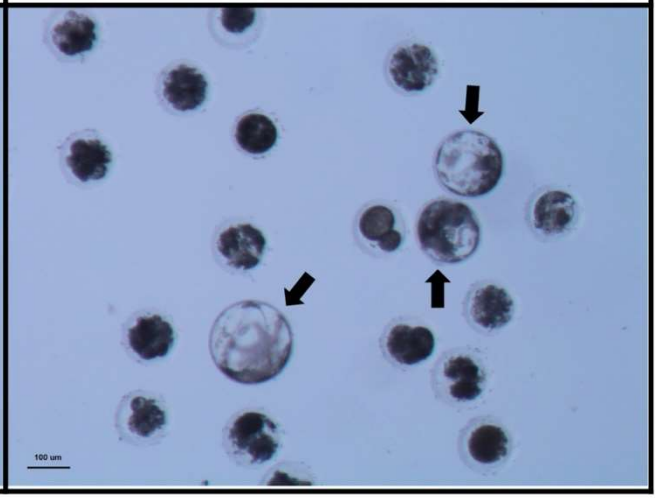
Source of oocytes	Embryos derived from oocytes with standard IVF	Embryos derived from oocytes with mitochondrial diffused pattern
Pre-pubertal		

Figure 3.2: Blastocysts (day 7; arrows) derived from pre-pubertal gilts oocytes with diffused mitochondrial pattern vs. standard IVF (control). The scale bar represents 100 µm. The arrows indicate to the blastocysts. The number of good quality (quality code 1) embryos was greater in the diffused group in comparison to standard IVF group.

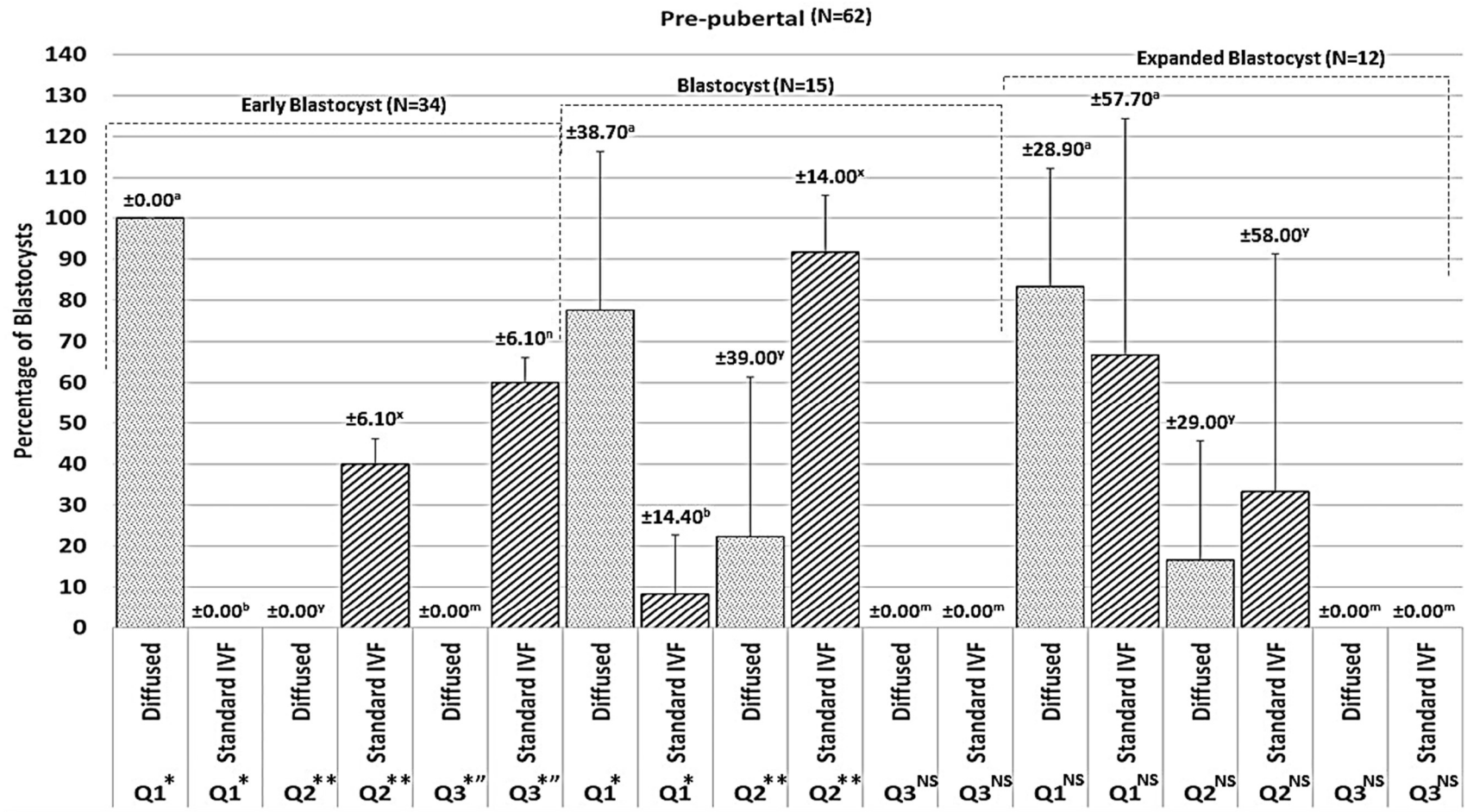


Figure 3.3: Quality of day 7 blastocyst derived from pre-pubertal gilts oocytes with diffused mitochondrial pattern vs. standard IVF (control). Q1-Q3 below each group name represent the blastocyst quality grades (1-3) according to the International Embryo Transfer Society. Grade 1 =Excellent or good quality; Grade 2 = Fair quality; Grade 3= poor quality.
* Within a quality (Q1) in the same blastocyst stage, values with different letters (a and b) are different (P≤0.05).
** Within a quality (Q2) in the same blastocyst stage, values with different letters (x and y) are different (P≤0.05).
*" Within a quality (Q3) in the same blastocyst stage, values with different letters (m and n) are different (P≤0.05).
(NS) indicates that there is no significant difference due to sorting within the same quality.

Sorting of oocytes based on cortical granule distribution and embryo development:

Standard IVF (69% ± 9.00; 105/153) and peripheral (81% ± 2.89; 113/142) groups had significantly higher cleavage rate than the other groups (P≤0.05). Cortical, intermediate, and unclassified groups had cleavage rates 33% ± 3.46 (18/51), 57% ±7.23 (35/65), and 53% ± 5.03 (23/44), respectively; these rates were different from the negative controls (P≤0.05). The peripheral group had higher (P≤0.05) morula rate (38% ± 5.20; 49/142) compared to other groups (Figure 3.4). The standard IVF group had an average morula rate of 27% ± 7.37 (36/153), and it was higher than intermediate (15% ± 3.61; 9/65), cortical (0% ± 3.46; 1/51), and unclassified (7% ± 6.51; 3/44) groups (P≤0.05). Intermediate and unclassified groups yielded morula rates approximately 10%, which was higher than negative controls (P≤0.05). The cortical group did not produce a significant number of morulae (0% ± 3.46; 1/51), similar to negative controls (0% ±0.00; 0/90; P≥0.05). At the blastocyst stage, only standard IVF (7% ± 3.51; 10/153) and peripheral (14% ± 1.53; 19/142) groups yielded blastocysts, and the peripheral group had a rate higher than the standard IVF group (P≤0.05). Also, the peripheral group generated 100% ± 0.00 good quality early blastocysts that was statistically (P≤0.05) greater than the standard IVF group (0% ± 0.00; Figure 3.5 and Figure 3.6).

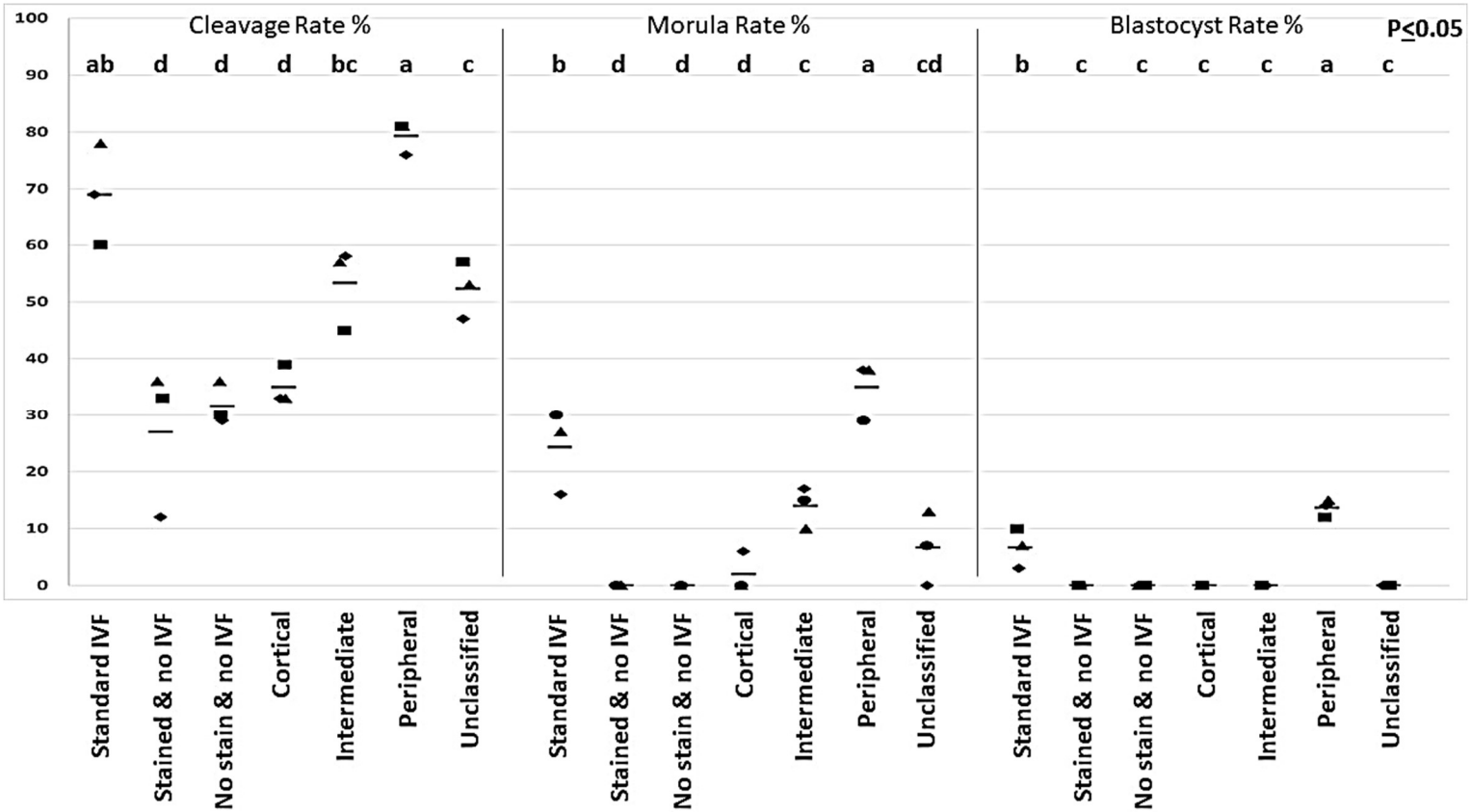


Figure 3.4: Fertilizing ability and embryo development of pre-pubertal gilts oocytes, sorted on the basis of distribution of cortical granules. Each group was evaluated for the cleavage (day 3), morula (day 5), and blastocyst (day 7) rates. Oocytes stained with PNA were classified as: cortical, intermediate, peripheral, and unclassified. Control groups were: standard IVF without staining, oocytes stained & no IVF, and no stain & no IVF. Different letters within the same embryo development stage indicates a significant difference (P≤0.05). The mean of the average rate was represented by the horizontal bar for each group.

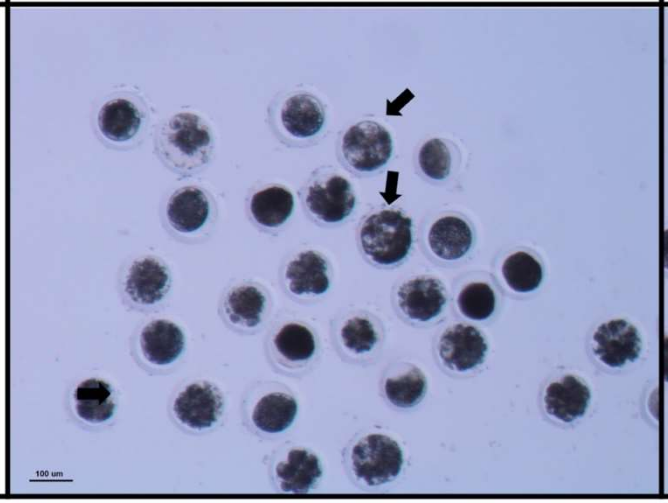
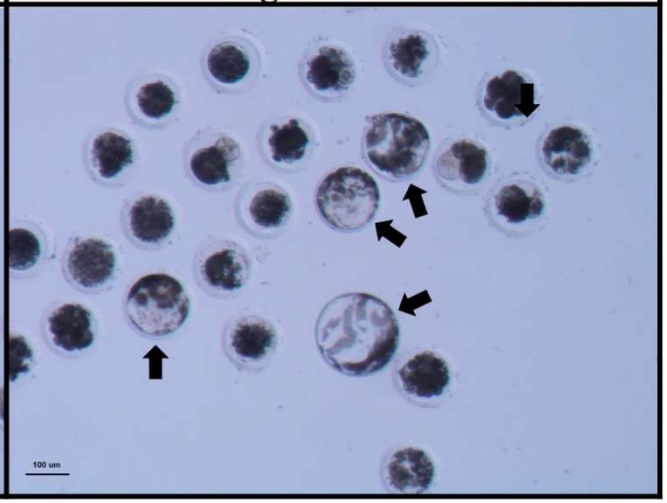
Source of oocytes	Embryos derived from oocytes with standard IVF	Embryos derived from oocytes with peripheral pattern of cortical granules
Pre-pubertal		

Figure 3.5: Blastocysts (day-7; arrows) derived from pre-pubertal gilts oocytes with peripheral cortical granules pattern vs. standard IVF (control). The scale bar represents 100 µm. The arrows indicate to the blastocysts. The number of a good quality (quality code 1) of embryos represented in peripheral group in comparison to standard IVF group is higher.

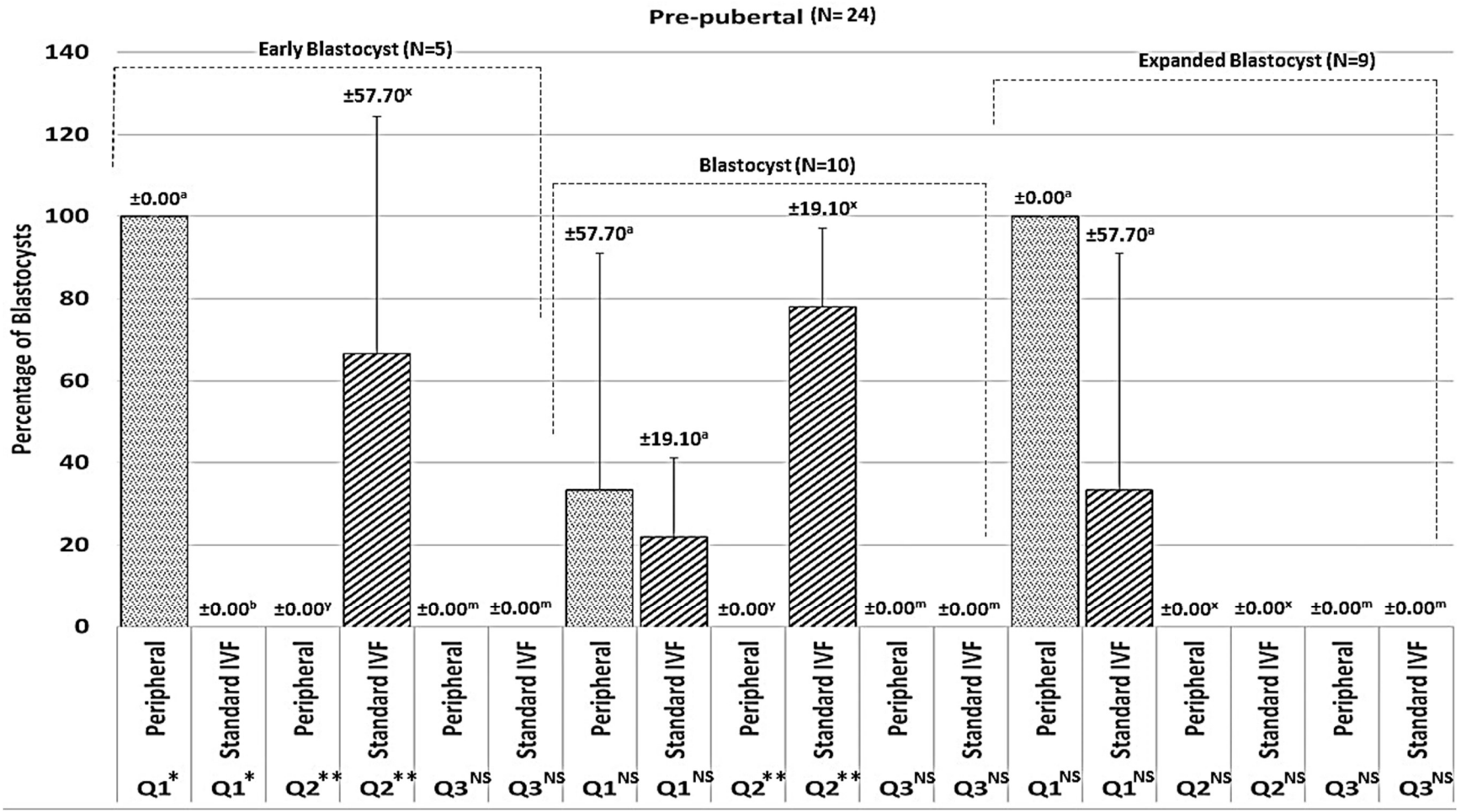


Figure 3.6: Quality of day-7 blastocyst derived from pre-pubertal gilts oocytes with peripheral cortical granules pattern vs. standard IVF (control). Q1-Q3 below each group name represent the blastocyst quality grades (1-3) according to the International Embryo Transfer Society. Grade 1 =Excellent or good quality; Grade 2 = Fair quality; Grade 3= poor quality.
* Within a quality (Q1) in the same blastocyst stage, values with different letters (a and b) are different (P≤0.05).
** Within a quality (Q2) in the same blastocyst stage, values with different letters (x and y) are different (P≤0.05).
(NS) indicates that there is no significant difference due to sorting within the same quality.

3.5.1.2. *In vitro* production and quality of pig embryos using sorted oocytes from adult sows

Sorting of oocytes based on mitochondrial distribution and embryo development:

Standard IVF and diffused groups showed 83% ± 4.16 (70/85) and 87% ± 3.63 (91/107) of the cleavage rate, respectively, which was significantly higher than other groups (P≤0.05; Figure 3.7). Semi-Peripheral (64% ± 7.38; 22/37) group had a higher cleavage rate than in negative control (24% ± 6.66; 19/84), peripheral (40% ± 21.81; 12/31), and unclassified (0% ± 29.96; 2/3) groups (P≤0.05). At morula stage, the standard IVF (47% ± 7.67; 38/85) and diffused (46% ± 3.81; 50/107) group had higher morula rate compared to the other groups (P≤0.05; Figure 3.7) but did not differ from each other. Also, the semi-peripheral group had higher morula rate (17% ± 8.01; 6/37) compared to negative (0% ± 0.00; 0/85), peripheral (0% ± 0.00; 0/31), and unclassified (0% ± 0.00; 0/3) groups (P≤0.05). At the blastocyst stage, only the standard IVF (12% ± 3.56; 10/85) and diffused (16% ± 4.72; 15/107) groups had blastocysts (Figure 3.7), and the diffused group had a slightly higher blastocyst rate than the standard IVF group. In addition, the diffused group had significantly (P≤0.05) more early blastocysts and blastocyst stage (100% ± 0.00) compared to the standard IVF group (0% ± 0.00 and 17% ± 19.10, respectively; Figure 3.8 and Figure 3.9).

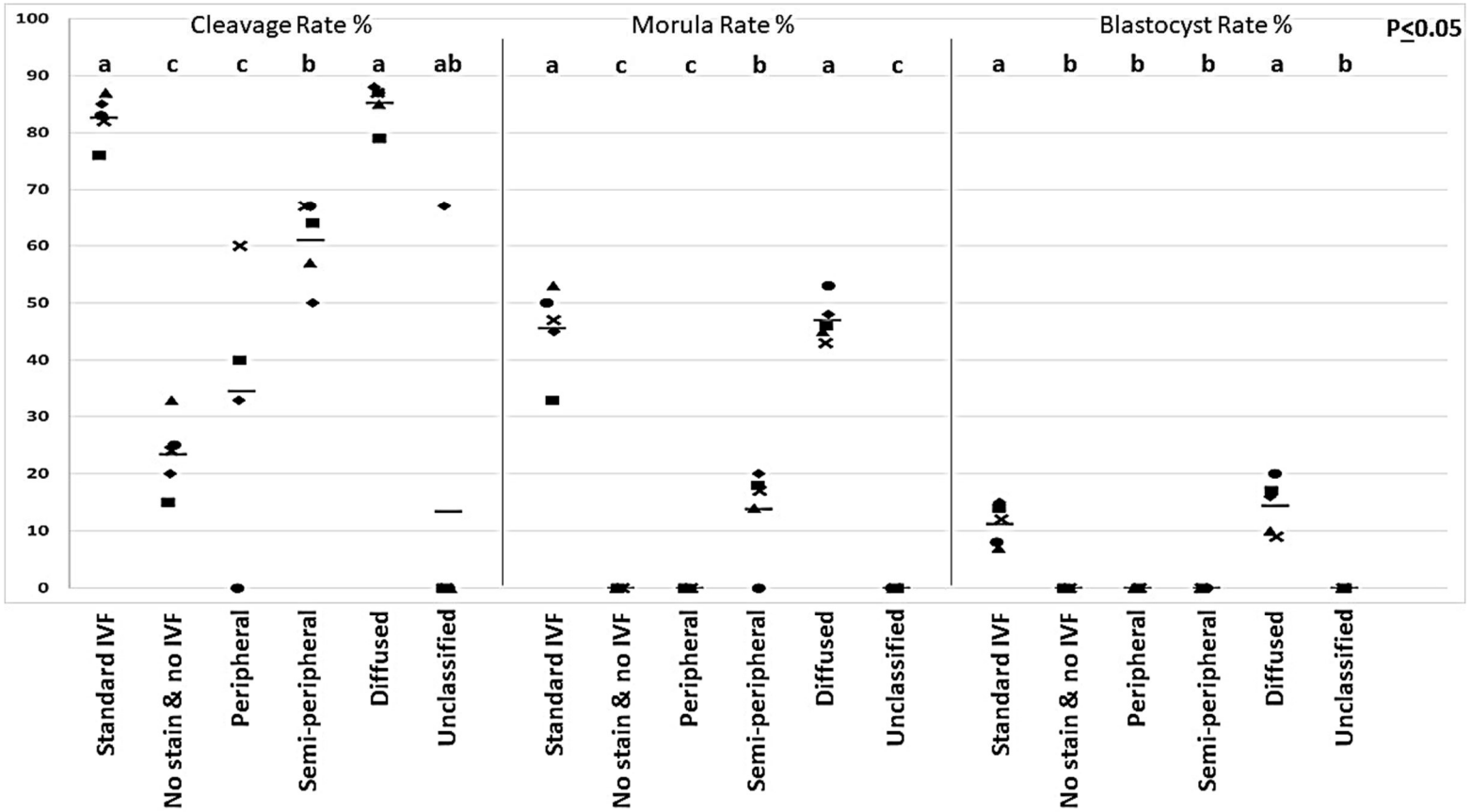


Figure 3.7: Fertilizing ability and embryo development of adult sow oocytes, sorted on the basis of distribution of mitochondria. Each group was evaluated for the cleavage (day-3), morula (day-5), and blastocyst (day-7) rates. Oocytes stained with MTG were classified as: peripheral, semi-peripheral, diffused, and unclassified. Control groups were: standard IVF without staining), oocytes stained but no IVF, and no stain and no IVF. Different letters within the same embryo development stage indicates a significant difference ($P \leq 0.05$). The mean of the average rate was represented by the horizontal bar for each group.

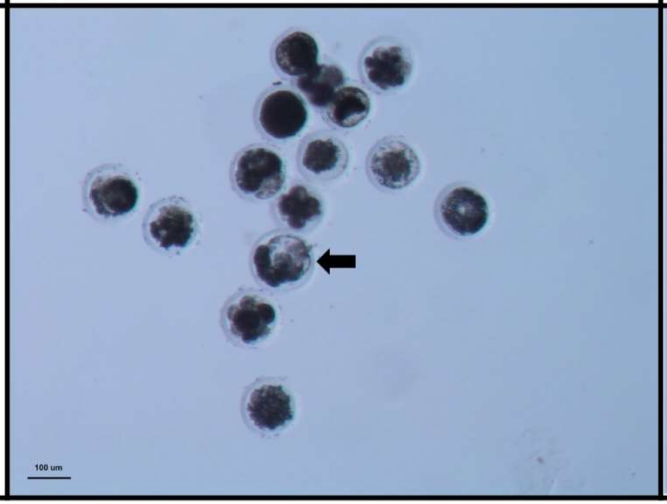
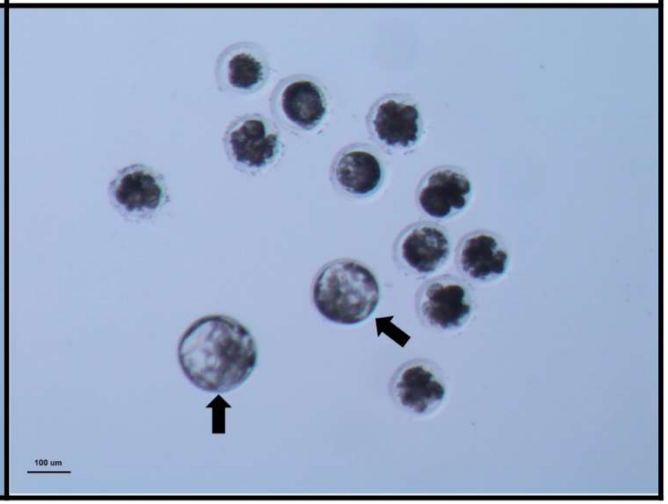
Source of oocytes	Embryos derived from oocytes with standard IVF	Embryos derived from oocytes with mitochondrial diffused pattern
Sow		

Figure 3.8: Blastocysts (day-7; arrows) derived from adult sow oocytes with diffused mitochondrial pattern vs. standard IVF (control). The scale bar represents 100 µm. . The arrows indicate to the blastocysts. The number of a good quality (quality code 1) of embryos represented in diffused group in comparison to standard IVF group is higher.

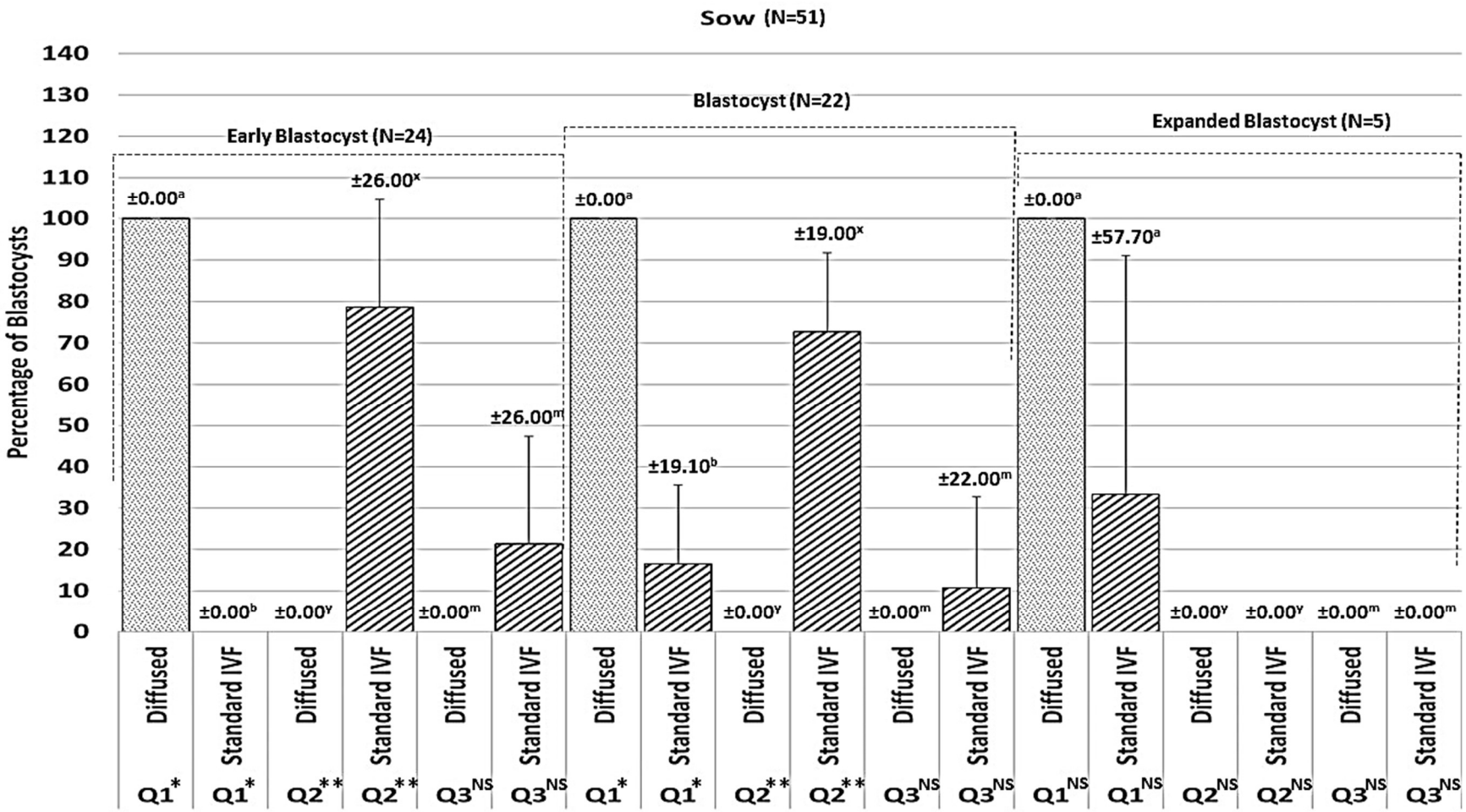


Figure 3.9: Quality of day-7 blastocyst derived from adult sow oocytes with diffused mitochondrial pattern vs. standard IVF (control). Q1-Q3 below each group name represent the blastocyst quality grades (1-3) according to the International Embryo Transfer Society. Grade 1 =Excellent or good quality; Grade 2 = Fair quality; Grade 3= poor quality.
* Within a quality (Q1) in the same blastocyst stage, values with different letters (a and b) are different (P≤0.05).
** Within a quality (Q2) in the same blastocyst stage, values with different letters (x and y) are different (P≤0.05).
(NS) indicates that there is no significant difference due to sorting within the same quality.

Sorting of oocytes based on cortical granules distribution and embryo development:

The standard IVF and peripheral groups had a higher cleavage (85 ± 2.63; 73/87, and 82% ± 6.95; 96/120, respectively) compared to the other groups (Figure 3.10, $P \leq 0.05$). The cleavage rate in intermediate group (56% ± 4.20; 20/36) was significantly higher than negative control (34% ± 4.43; 29/84), cortical (39% ± 5.56; 13/35), and unclassified (79% ± 26.87; 5/7) groups ($P \leq 0.05$). Interestingly, the cleavage rate in cortical group did not differ with the negative control ($P \geq 0.05$). The unclassified group had a wide range of cleavage rate to the other groups. At the morula stage, only standard IVF (38% ± 6.24; 33/87), peripheral (40% ± 3.77; 47/120), and intermediate (23% ± 4.97; 8/36) groups produced morulae. Standard IVF and peripheral groups had the highest morula rate ($P \leq 0.05$) compared to the other groups. At the blastocyst stage, only standard IVF (15% ± 4.16; 13/87) and peripheral (16% ± 4.92; 19/120) groups produced blastocysts and their rates were similar ($P \geq 0.05$). Also, the peripheral group had better quality ($P \leq 0.05$) of early and expand blastocysts (100% ± 0.00 and 81% ± 17.20, respectively) compared to the standard IVF group (0% ± 0.00; Figure 3.11 and 3.12).

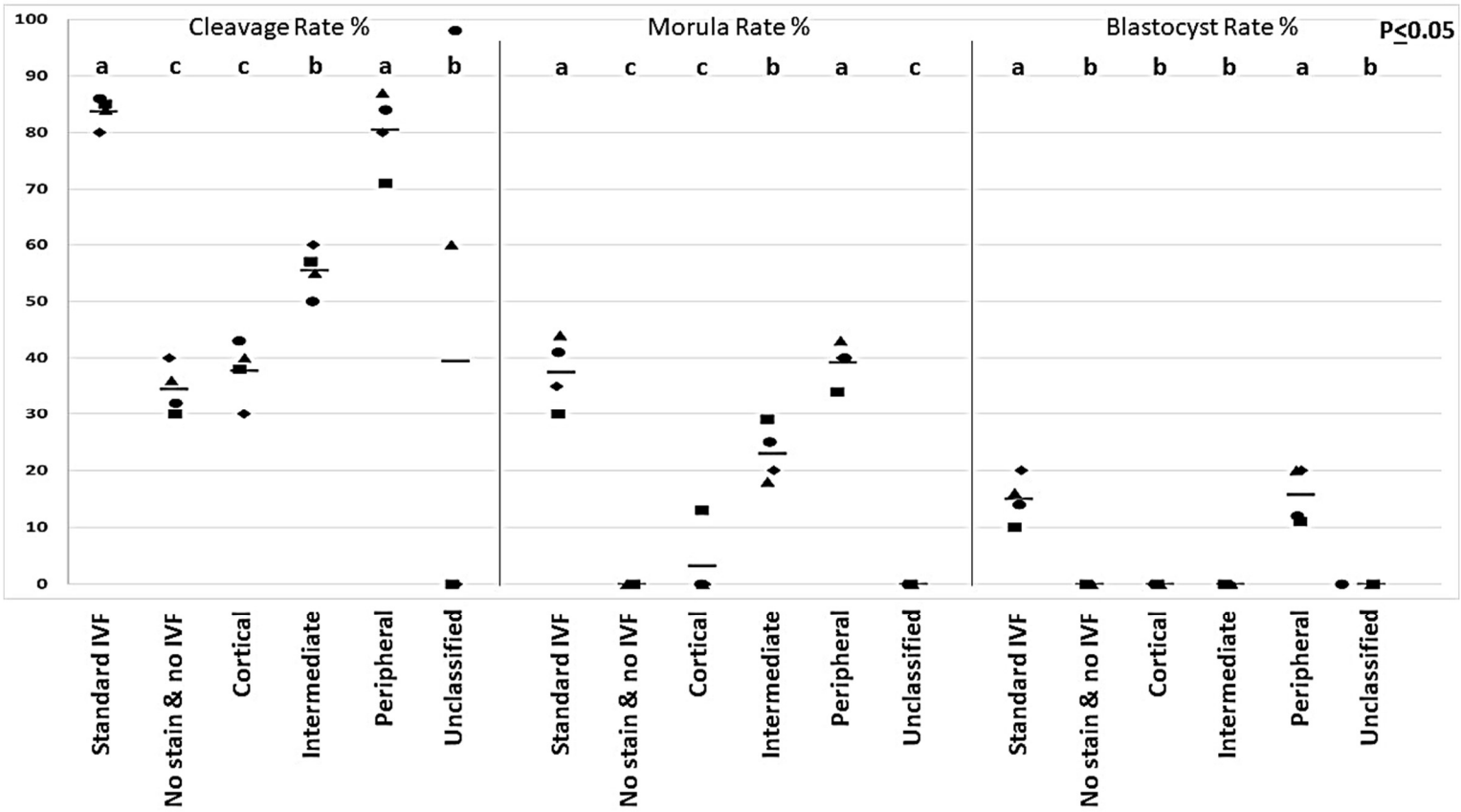


Figure 3.10: Fertilizing ability and embryo development of adult sow oocytes, sorted on the basis of distribution of cortical granules. Each group was evaluated for the cleavage (day 3), morula (day 5), and blastocyst (day 7) rates. Oocytes stained with PNA were classified as: cortical, intermediate, peripheral, and unclassified. Control groups were: standard IVF without staining, oocytes stained but no IVF, and no stain and no IVF. Different letters within the same embryo development stage indicates a significant difference ($P \leq 0.05$). The median of the average rate was represented by the horizontal bar for each group.

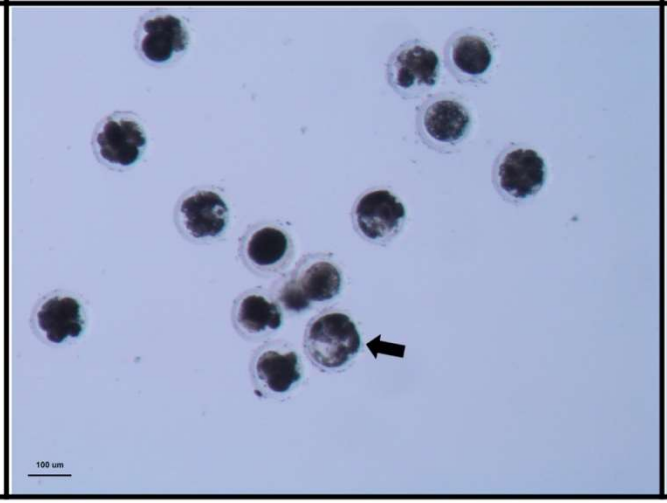
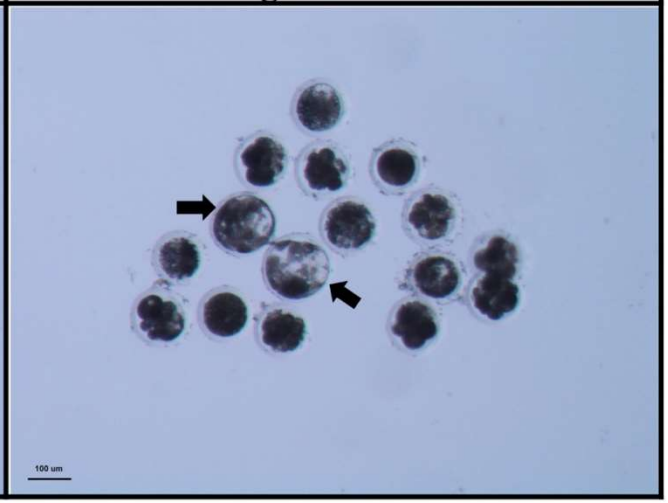
Source of oocytes	Embryos derived from oocytes with standard IVF	Embryos derived from oocytes with peripheral pattern of cortical granules
Sow		

Figure 3.11: Blastocysts (day-7; arrows) derived from adult sow oocytes with peripheral cortical granules pattern vs. standard IVF (control). The scale bar represents 100 µm. The arrows indicate to the blastocysts. The number of a good quality (quality code 1) of embryos represented in peripheral group in comparison to standard IVF group is higher.

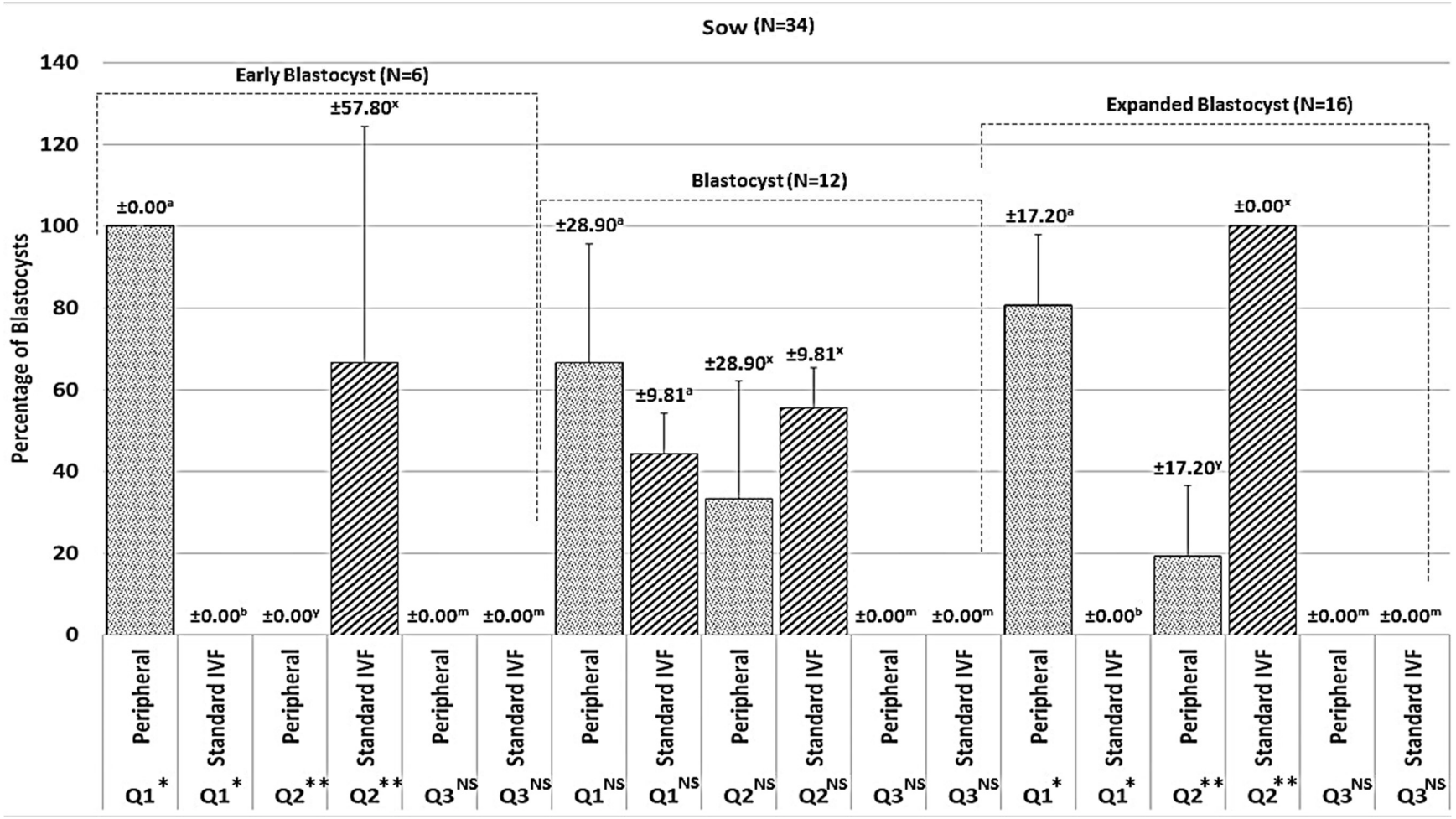


Figure 3.12: Quality of day-7 blastocyst derived from adult sow oocytes with peripheral cortical granules pattern vs. standard IVF (control). Q1-Q3 below each group name represent the blastocyst quality grades (1-3) according to the International Embryo Transfer Society. Grade 1 =Excellent or good quality; Grade 2 = Fair quality; Grade 3= poor quality.
* Within a quality (Q1) in the same blastocyst stage, values with different letters (a and b) are different (P≤0.05).
** Within a quality (Q2) in the same blastocyst stage, values with different letters (x and y) are different (P≤0.05).
(NS) indicates that there is no significant difference due to sorting within the same quality.

3.5.2. Experiment 2: Evaluation of Polyspermy in Pig Embryos from Sorted Oocytes Based on Cortical Granules Distribution

Due to limited access to sow ovaries, only the pronuclei after fertilization of pre-pubertal oocytes were counted. Oocytes having peripheral CGs had the highest monospermic fertilization (64% ± 5.20; 57/89) compared to the other groups (Table 3.2). Also, the peripheral group had the lowest polyspermic fertilization (12% ± 1.53; 11/89) compared to the other groups. Oocytes from cortical group (considered "immature") had the lowest monospermic fertilization (14% ± 0.58; 8/56) and the highest rate of polyspermic fertilization (41% ± 2.89; 23/56; Table 3.2).

Table 3.2: Polyspermy counts (rates) by cortical granule distribution pattern. Each number represents the mean (%) ± SD.

Pattern	Total # of oocytes	Fertilization status			
		Unfertilized (**)	Normal fertilized zygotes (**)	Polyspermic fertilized oocytes (**)	Unknown (NS)
Standard IVF *	181	20 ± 9.54 a (w) (N=36) [34,13,10]	50 ± 1.00 b (w) (N=90) [45,56,50]	28 ± 3.06 a (w) (N=50) [21,31,32]	3 ± 2.89 c (w) (N=5) [0,0,8]
Cortical *	56	41 ± 4.04 a (x) (N=23) [56,53,16]	14 ± 0.58 b (z) (N=8) [11,16,16]	41 ± 2.89 a (x) (N=23) [33,32,58]	5 ± 1.73 c (w) (N=3) [0,0,16]
Intermediate *	65	29 ± 0.58 a (wx) (N=19) [30,35,24]	38 ± 0.58 a (wy) (N=25) [40,45,32]	28 ± 2.00 a (w) (N=18) [30,20,32]	8 ± 2.89 b (w) (N=5) [0,0,20]
Peripheral *	89	21 ± 3.79 a (w) (N=19) [25,24,11]	64 ± 5.20 b (x) (N=57) [61,65,68]	12 ± 1.53 ac (y) (N=11) [14,12,11]	2 ± 1.15 c (w) (N=2) [0,0,11]
Unclassified *	67	36 ± 3.61 a (wx) (N=24) [38,39,27]	36 ± 4.58 a (y) (N=24) [41,39,20]	21 ± 1.53 ab (wy) (N=14) [21,22,20]	7 ± 2.89 b (w) (N=5) [0,0,33]

* Within a pattern (row), values with different letters (a-c) are different (P≤0.05).
** Within a pronuclei number (column), different letters (w-z) represent differences among the patterns (P≤0.05).
(NS) indicates that there is no significant difference due to patterns.
N represents number of zygotes used.
The numbers between square brackets represent the percentage of each replicate.
Unclassified pattern refers to no stained pronucleus.
Unknown refers to poor signal of DAPI to recognize the number of pronuclei.

3.6. Discussion

Our study demonstrated that it is possible to sort pig oocytes based on their cytoplasmic maturation (MT or CGs distributions using MitoTracker Green and Peanut Agglutinin respectively) to produce embryos after *in vitro* fertilization and culture. However, the blastocyst rate was statistically similar between sorted (based on diffused group in MT and peripheral CGs; indications of oocytes maturation) and the standard IVF group. Thus, our hypothesis was partially supported. The sorting of oocytes did not improve the production of embryos rate in cleavage stage and morula stage, but it improved the blastocyst stage from sorted oocytes derived from pre-pubertal gilts as well as the gross quality of embryos. The blastocyst rate in this study was lower compared to previous reports (ISOM et al., 2012, GIL et al., 2013, MARTINEZ et al., 2015a), however, the quality of the produced blastocyst was also better for the sorted group compared to the standard IVF group. Interestingly, the quality of blastocyst did not increase in oocytes sorted based on lipid location; however, the number of produced blastocyst increased (HIRAGA K et al., 2013). This study reported an increase of blastocyst by comparing immature vs. mature oocytes but did not compare *in vitro* production of blastocyst from non-sorted oocytes. Regardless, improving the quality of *in vitro* produced blastocyst would be beneficial for studies requiring healthy pig embryos.

In general, oocytes used in IVEP can be harvested from slaughtered pre-pubertal gilts or adult sows. These slaughtered pigs were in different estrous cycle stages; thus, harvested oocytes were at different maturation stages. Regardless of maturation process (chapter 2), CG-sorted oocytes from both sources generated the same rate of blastocysts.

This study demonstrated that when oocytes are grouped based on the location of MT or CGs, there is an improvement in the quality of *in vitro* produced embryos. However, the production of blastocysts did not increase. The overall success rate of *in vitro* fertilization of pig oocytes has historically been very challenging (FOWLER et al., 2018). Our subjective evaluation revealed a higher number of produced blastocysts with grade 1 quality compared to the standard IVF group. It is known that grouping embryos would increase interactions among them and improve their development (PARIA and Dey, 1990). Assuming that our sorting method will group oocytes of similar cytoplasmic maturation stage, the embryos produced will have the similar autocrine/paracrine factors required for maturation and embryonic development

(WYDOOGHE et al., 2017, MACHTINGER et al., 2016). These interactions could be positive and beneficial for the growth of embryos. For instance, parthenogenetic embryos can increase the development of cloned embryos when they are co-cultured together (SAADELDIN et al., 2014). These interactions could also be detrimental. The degenerated embryos expressed different miRNA compared to healthy blastocyst have been found in the culture media which could affect healthy embryos (KROPP et al., 2014). So, we can hypothesize that regrouping oocytes with similar MT or CGs location will generate a unique environment that could positively or negatively influence the development of embryos. In this study, the improvement of blastocyst quality from the sorted (matured) group is possibly due to harmful autocrine/paracrine factors were removed from oocytes by our sorting method.

Except for pre-pubertal oocytes, oocytes sorted based on MT location had a significantly better production of blastocysts than sorted based on the location of CGs. This suggests that mitochondrial diffusion is a reliable criterion for oocyte maturation as compared to CG distribution. However, this proportion of produced blastocysts should have been higher if all oocytes of this group were considered having a "matured" cytoplasm. The embryo-to-embryo interaction during *in vitro* culture influences embryonic growth (STOKES et al., 2005). In the present study, an *in vitro* production (culture) system was used with a fixed volume, regardless of the number of embryos. Also, our sorting method reduced the total number of oocytes available for our IVEP system (range 10 to 50 oocytes per 500µl; data not shown). The final volume of media was calculated as 10-13 µl/oocyte in standard IVF group and sorted matured groups derived from pre-pubertal gilts, and 17-29 µl/oocyte in adult sow oocytes. On the other hand, there was more than 17 µl/oocyte for the other groups (stained & no IVF, no stain & no IVF, and sorted unmatured oocytes) based on pre-pubertal gilts and more than 24 µl/oocyte based on adult sow oocytes. So, the distance between embryos was greater for some of the groups and the positive environment produced by healthy embryos was likely reduced and the growth of the companion embryos was likely affected. Another possibility is that oocytes considered matured based on our sorting technique may not be sufficiently mature or competent to sustain the growth of embryos. Only two markers (MT and CGs) were used to recognize a cytoplasmic maturation of oocytes. So, it may require more markers to isolate the fully matured oocytes. Some embryos were arrested at different stages (two cells, four cells, or eight cells) which might be due to lack of fertilization because of high concentration of reactive oxygen

species (BEDAIWY et al., 2004), parthenogenesis (SFAKIANOUDIS et al., 2021), embryo genetics especially maternal genes (LONGO et al., 2018), or insufficient energy in culture medium (HARDY et al., 2001). The previous factors of embryo arrest may be an explanation to low rate of embryos development to morula or blastocyst stages in our study. The arrest of embryonic development at 8 cell stage is mainly due to the lack of zygote activation (SUN et al., 2019). Finally, we cannot discard the possibility that these arrested embryos may secrete negative paracrine factors, that influence the growth of other embryos.

Polyspermy is one of the main factors affecting the production of porcine embryos (GRUPEN, 2014). Several studies reported reductions in the polyspermy rate by controlling sperm concentration (COY et al., 1993a), adding protein (SAAVEDRA et al., 2014), developing new maturation media (ROMAR et al., 2016), and adding seminal or oviduct fluid to maturation medium (BATISTA et al., 2016). A majority of these studies used a mixture of oocytes matured at different stages which could influence the polyspermy rate. In this study, the polyspermy was studied in the oocytes sorted based on the location and distribution of CGs, providing an advantage to evaluate the exocytosis of CGs based on the status of maturation. The results of this study revealed that polyspermy is mainly associated with non-sorted (non-matured) oocytes and sorted (matured) oocytes had greater ability to control the number of sperm entering into oocytes (see Table 3.2). The variation among the replicates in this experiment may be due to many reasons. For example, semen quality, age of animals, temperature during transportation, weather etc. Thus, we recommend sorting matured from non-matured oocytes to minimize the negative influence of the polyspermy on monospermic zygotes during the embryo development.

Our experiments had only 3 replicates in pre-pubertal oocytes and tested several treatments, so the results should not be considered definitive. They might suggest trends that could be followed up with additional experiments with more replicates and fewer treatments to confirm. Also, all of the ANOVA tests done within a table were inter-related because the percentages in one cell affect multiple ANOVAs. This is a weakness of the statistical analyses. If there were many more replicates, a more sophisticated statistical analyses could be done.

3.7. Conclusions

There are several important conclusions from this study. First, the sorting of oocytes based on MT or CGs location will produce embryos of better quality for cryopreservation or embryo transfer into recipients compared to non-sorted oocytes. Second, polyspermy rates in sorted-matured oocytes would be reduced when applied in IVF. Finally, variation in the embryo production will be minimized by having all oocytes on the same maturation stage during *in vitro* fertilization. Potentially, it could also minimize the variation in studies requiring an embryo response after treatment. Both MTG and PNA markers are useful for the sorting of mature oocytes. We were unable to improve the number of blastocysts after sorting oocytes based on MT or CGs. *In vitro* fertilization rates in pig are generally low as compared other domestic animals (FOWLER et al., 2018). Finally, polyspermy can be decreased using CGs sorting with peripheral pattern. Additional studies are required to investigate new cytoplasmic markers and to understand the different mechanism leading to cytoplasmic maturation of the pig oocyte.

CHAPTER 4: THE EFFECT OF SORTING OOCYTES AND TYPES OF MATURATION MEDIA ON PIG EMBRYO PRODUCTION *IN VITRO* AND POLYSPERMY

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Relationship of this study to the dissertation

In chapter 4, the ability of maturation media on sorted oocytes to produce embryo and polyspermy was evaluated. In this study, oocytes were sorted based on mitochondrial and cortical granules distributions (as in chapter 2). In addition, we used sorted oocytes using cortical granules distribution to evaluate cleavage, morula, and blastocysts rates using different media. In this study we hypothesized that different maturation media have different effect on cortical granules and mitochondrial distribution, *in vitro* embryo production (IVEP), and polyspermy rates in pigs.

Authors' Contributions

Hamza Al-Maamory: Perform the study, experimental design, write the manuscript.
Carl Lessard: Supervised and participated in the experimental design and critical revision the manuscript.

4.1. Abstract

A lack of cytoplasmic maturation may result in decreased effectiveness of the *in vitro* embryo production (IVEP) in porcine. Distribution and patterns of mitochondrial (MT) and cortical granules (CGs) are related to the cytoplasmic maturation of oocytes. Many maturation media are commercially available to mature the pig oocytes. We hypothesize that different maturation media for pig oocytes have different effects on the distribution of CGs and MT, embryo development, and polyspermic fertilization in porcine. The specific objectives of this study were to evaluate: 1) differences in MT and CGs distribution in the oocytes collected before and after the maturation from pre-pubertal pigs; 2) the ability of sorted oocytes, based on CGs' distribution, to maintain embryo development *in vitro*; and 3) to assess polyspermia using the pronuclear test regarding the distribution of CGs of oocytes matured under different media. Grade-1 and -2 oocytes were collected from pre-pubertal gilts. Oocytes were matured *in vitro* using different maturation media (TCM, Tissue Culture Medium-199; mWM, modified Whitten's Medium; NCSU23, North Carolina State University 23; NCSU37G, North Carolina State University 37 with glucose; and NCSU37PL, North Carolina State University 37 with pyruvate and lactate) under 5% CO_2, 5% O_2 and 90% N_2 in a humidified environment, and at 38.5˚C for 44 hr. Cumulus cells were denuded, and the oocytes stained with 200 nM of MitoTracker Green (MTG) and 625 nM of Peanut Agglutinin coupled with FITC (PNA) to evaluate MT and CGs distribution, respectively. The stained oocytes were separated based on three patterns of MT (peripheral, semi-peripheral, and diffused), and three patterns of CGs (cortical, intermediate, and peripheral) using wide-field microscope. Only CGs sorted oocytes were used in the rest of the study and fertilized separately using the standard *in vitro* fertilization (IVF) procedure. Cleavage, morula, and blastocyst rates as well as the pronuclear test were evaluated. After 44 hr of maturation, differences in the maturation rate were evident with diffused MT pattern significantly greater in TCM (55% ± 1.00; 82/150) and NCSU37PL (51% ± 1.53; 77/150) groups compared to NCSU23 (40% ± 2.00; 60/150) and NCSU37G (43% ± 0.58; 64/150) groups ($P \leq 0.05$). Likewise, the oocyte maturation rate based on peripheral pattern of CGs was higher ($P \leq 0.05$) in TCM (60% ± 3.00; 90/150) than mWM (46% ± 3.60; 69/150) or NCSU23 (42% ± 2.60; 63/150). The overall cleavage, morula and blastocyst rates ranged from 62% to 77%, 20% to 31% and 10% to 15% respectively, in all media with sorted matured

oocytes. NCSU37PL media with pyruvate supplement demonstrated lower polyspermy rate compared to other media (respectively 22% ± 3.20; 85/381; versus 26-33%). In comparison to other groups, there was a significant decrease in polyspermy of oocytes with the peripheral pattern ($P \leq 0.05$). In summary, this study demonstrated that oocytes matured in pyruvate base media had more MT diffused pattern and more CGs peripheral pattern than other media. However, the cleavage, morula and blastocyst rates did not differ between media. In addition, the pyruvate-based media decreased the polyspermy rate.

4.2. Introduction

Oocyte maturation is important in successful *in vitro* embryo production (IVEP). The major factor important in porcine oocyte *in vitro* maturation (IVM) is maturation medium used. The main factors in media that affect oocytes' maturation are the anti-oxidant content, oxygen tension, and supplement or chemical ingredients in media (NAGAI et al., 2006). Fetal calf serum (FCS), gonadotropins (ABEYDEERA, 2002), porcine follicular fluid (pff), and various hormones are commonly used as supplements in IVM media (ZHANG et al., 2012).

Researchers have been working on developing IVM strategies. The first successful IVM was achieved using Difco's medium-199 (EDWARDS, 1965). The base component of this maturation media remained without any change for about 40 years (THOMPSON and GILCHRIST, 2013). Since 2000s, there were many attempts to reach the successful *in vivo* maturation on *in vitro* conditions (DE VOS et al., 2021). There are numerous maturation media available which can be used, but selecting the base or the best medium for IVM is believed to be very challenging (KIM et al., 2011). Currently, North Carolina State University (NCSU) medium, modified tissue culture medium (TCM) 199 and modified Tyrode's medium containing lactate and pyruvate (mTLP) supplemented the porcine follicle fluid (pff) or serum with the three previous media are commonly used for pig IVM (ZHANG et al., 2012). In fact, pff was found to improve the nuclear and cytoplasmic maturation of oocytes (GRUPEN, 2014). Later, North Carolina State University (NCSU) 37 medium (with pyruvate and lactate), North Carolina State University (NCSU) 37 medium (with glucose), and modified Whitten's Medium (mWM) were used in the maturation process (FOWLER et al., 2018). It is necessary that the maturation medium supplies the energy molecules based on the glycolytic pathway to the oocytes during IVM, such as pyruvate and adenosine triphosphate (ATP) (DE ARAUJO et al., 2009). Therefore, it is important to provide the media with pyruvate (KIM et al., 2011). Many researchers have suggested adding luteinizing hormone (LH), follicle stimulating hormone (FSH) (XIAO et al., 2014, PANDEY et al., 2010), epidermal growth factor (EGF; (WANI et al., 2012)), and cysteine (GASPARRINI et al., 2006) to improve the maturation process.

Our objectives were to: 1) evaluate the effects of different maturation media on mitochondrial (MT) and cortical granules (CGs) patterns of pig oocytes; 2) study the effect of different maturation media on IVEP outcome; and 3) determine the effect of different maturation

media on the polyspermy in pig embryos. We hypothesized that different maturation media will demonstrate differences in CGs and MT distribution, IVEP, and polyspermy in pigs.

4.3. Materials and Methods

4.3.1. Animals and chemicals

Oocytes used in this experiment were derived from pre-pubertal gilts and adult sows ovaries brought to a slaughterhouse near Saskatoon. All chemicals were used from Sigma-Aldrich Canada Co. (Oakville, Ontario, Canada), unless otherwise mentioned. All media were filtered with a 0.22 µm PVDF filter (Argos technology, Elgin, USA) for this study.

4.3.2. Oocytes Collection

Ovaries, collected from abattoir, were placed into an insulated container (35-37 ºC) for transport to the Westgen Lab, University of Saskatchewan. After removal of surrounding non-ovarian connective tissues, ovaries were washed (3X) in sterile saline (0.9% NaCl, at 22 °C) and immersed in glucose solution (0.128 M, at 22 °C) for 30 min. Following this, cumulus–oocyte-complexes (COCs) were harvested from three to eight-mm (diameter) follicles. Grade I and II COCs were washed (3X) with washing medium (TCM-199, Invitrogen, Burlington, Canada; supplemented with 25 mM of HEPES and 50 mg/ml gentamycin; maintained at 37 ˚C).

4.3.3. Oocyte Maturation

The COCs were rinsed once in their respective maturation media: Tissue Culture Medium (TCM)-199 as a control, North Carolina State University 23 (NCSU23), modified Whitten's Medium (mWM), North Carolina State University 37 with glucose (NCSU37G), and North Carolina State University 37 with pyruvate and lactate (NCSU37PL). All previous listed media were prepared in our lab except the TCM media (Invitrogen, Burlington, Canada). The composition of different media used in this study are presented in Table 4.1. Subsequently, COCs (maximum 50/well) were transferred into a 4-well dish containing 500 µl of maturation

medium/well and incubated for 44 hrs under a controlled environment (5% CO_2, 5% O_2 and 90% N_2 in a humidified environment, and at 38.5˚C). Before maturation, a portion of oocytes were denuded using 0.1% hyaluronidase in TCM-199, with repeated pipetting (approximately 80 times) using 200 µl-pipette set at 100 µl. Another portion of oocytes were denuded after 44 hrs of maturation using repeated pipetting with respective media. The denuded oocytes were washed twice with phosphate buffered saline (PBS; Thermo Fisher Scientific, Gibco, Canada) with 0.1 % Polyvinyl Alcohol (MARQUES et al., 2012) and then transferred into 50 µl droplets of 0.1% PVA-PBS buffer containing either 200 nM of MitoTracker Green (MTG, Invitrogen) or 625 nM of Peanut Agglutinin (PNA) coupled with fluorescein isothiocyanate (FITC). After an incubation in a controlled environment (90% N2, 5% CO2, and 5% O2 at 38.5 ˚C, in the dark.) for 30 min, stained oocytes were washed (3X) with 0.1% PVA-PBS buffer and transferred into the respective maturation medium.

4.3.4. *In Vitro* Fertilization

Oocytes were divided into several groups, depending on the source of oocytes, for *in vitro* fertilization (IVF) study: control 1 contained matured oocytes from standard procedure using TCM-199 media with 10% pff; control 2 contained matured oocytes in TCM-199 media with no fertilization or staining; and all other groups were the respective treatment groups. All groups are matured oocytes stained with the FITC-PNA within each media (mWM, NCSU23, NCSU37G, and NCSU37PL) as well as the stained groups within TCM-199. Subsequently, COCs (maximum 50 COCs/well) were transferred into a 4-well dish (500 µl of maturation medium/well), and incubated under a controlled environment (5% CO_2, 5% O_2 and 90% N_2 in a humidified environment at 38.5˚C) for 44 hrs. IVF was used to assess the ability of sorted oocytes to sustain embryo development. After the maturation period, oocytes were denuded with 0.1% hyaluronidase in TCM-199, with repeated pipetting as described above. A portion of oocytes remained unstained (control) and washed (3X) in a modified Tris-buffered medium (mTBM) supplemented with 0.2% bovine serum albumin (BSA; Minitube, Verona, USA) and 1 mM caffeine). The other portion of oocytes was stained with FITC-PNA and sorted as previously described. The washed and/or stained oocytes were transferred into mTBM fertilization medium. Frozen semen from an elite boar with proven fertility was used for fertilization. Semen straws

were thawed at 37°C for 20 seconds and diluted with mTBM media and centrifuged at 1000 × g for 5 min twice and resuspended in 1mL of mTBM for capacitation. Sperm motility and concentration were analyzed using a Computer Assisted Sperm Analyzer (Spermvision®, Minitube Canada). A total of 50,000 motile sperm cells were incubated with matured oocytes at 38.5°C in a controlled environment for 6 hrs.

Table 4.1: Chemical composition and concentrations (mM/L) of different maturation media for pig oocytes, used in this experiment.

Ingredients	TCM-control	NCSU23*	mWM*	NCSU37G*	NCSU37PL*
CaCl2	-	1.70	-	-	-
CaCl2.2H2O	*** 1.80	-	-	1.70	1.70
Calcium lactate	-	-	1.71	-	-
Cysteine	825.35	0.57	0.57	-	-
Fatty acid-free BSA (mg/ml)	-	-	-	4.00	4.00
Gentamycin (mg/ml)	50	-	-	-	-
Glucose	3.047 + *** 5.55	5.55	5.56	5.55	-
Hepes	** 25	-	-	-	-
Hypotaurine	-	5.00	-	-	-
KCl	*** 5.16	4.78	4.78	4.78	4.78
KH2PO4	-	1.19	1.19	1.19	1.19
L-Glutamine	** 0.5	1.00	-	1.00	1.00
MgSO4.7H2O	*** 1.66	1.19	1.19	1.19	1.19
NaCl	*** 116.39	108.73	68.49	108.73	108.73
NaHCO3	** 26.19	25.07	25.07	25.07	25.07
NaH2PO4.H2O	*** 1.02	-	-	-	-
Penicillin G	-	100.00	100.00	-	-
Polyvinyl alcohol (mg/ml)	22.699	-	-	-	-
Porcine follicular fluid (%, v/v)	10.00	10.00	10.00	-	-
Sodium lactate	-	-	25.20	-	2.73
Sodium pyruvate	0.909	-	0.33	-	0.17
Sorbitol	-	-	-	-	12.00
Streptomycin (µg/ml)	-	50.00	50.00	-	-
Taurine	-	7.00	-	-	-

* The chemical composition of each media (FOWLER et al., 2018) ** Part of medium 199 that we used. *** Earle's salts within medium 199 that we used. Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate)

4.3.5. Embryo Culture

Presumptive zygotes were washed (3X) using a modified NCSU23 (glucose was replaced by sodium lactate (4.5 mM) and sodium pyruvate (0.33 mM)), and then cultured in the same media (modified NCSU23) supplemented with 0.4% BSA in an environment of 5% CO_2, 5% O_2 and 90% N_2, for 48 hrs. After culture, embryos were transferred back into original NCSU23 (with 5.55 mM of glucose) for additional 5 days. Cleavage and embryo development rates were evaluated on day 3, 5, and 7 after fertilization.

4.3.6. Experiment 1: Determination of Maturation and Polyspermic Fertilization (Preliminary Experiment)

4.3.6.1. Determination of maturation of pig oocytes

A preliminary experiment was conducted to check the maturation stage of oocyte using anti-lamin antibody/DAPI staining. A total of 78 adult sow and 168 gilt oocytes, in 3 replicates, were matured in medium for 44 hr as described in section 4.3.3. After maturation, oocytes were stained with anti-lamin antibody and DAPI immunofluorescence procedures to determine germinal vesicles (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) stages (Figure 4.1). After 44 hr IVM, oocytes were denuded using hyaluronidase and transferred into 4-well plates. The denuded oocytes were fixed with 4% paraformaldehyde, sealed with parafilm, and kept in the refrigerator overnight. After the fixation, oocytes were transferred to 0.5% Triton X-100 in Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Burlington, ON, Canada) for 30 min for permeabilization. Following the permeabilization, the oocytes were transferred into 0.05% Tween 20 in DPBS solution for 30 min. The oocytes were washed (2X) with blocking buffer (2% BSA in PBS) for 5 min.

Oocytes were incubated with mouse anti-lamin stain (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer (1:300) for 1 h and washed (3X) in washing buffer (5 min each). Five oocytes were transferred through three 5-µL drops of Vectashield Mounting Medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) for 3X. The last droplet was mounted on a glass slide under a coverslip (using Vaseline:parafilm droplet at each corner of the

coverslip) and evaluated under epifluorescence microscope (Carl Zeiss Ltd., Toronto, ON, Canada) for nuclear maturation stage (PRENTICE-BIENSCH et al., 2012). The MII stage, characterized by two pronuclei, was considered as an indicator of the matured oocyte.

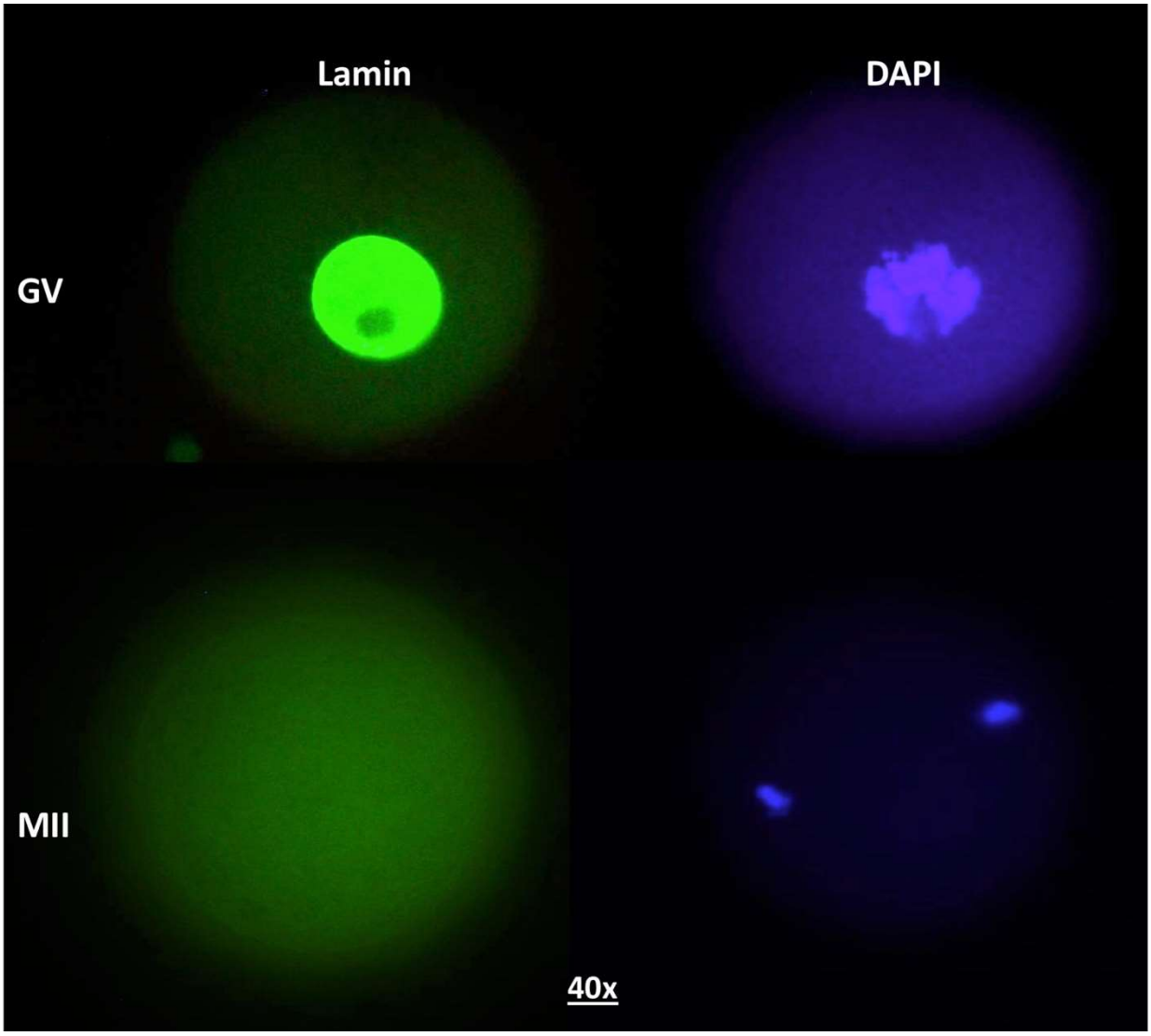

Figure 4.1: Classification of porcine oocytes in germinal vesicle (GV) and metaphase II (MII) stages using anti-lamin and 4',6-diamidino-2-phenylindole (DAPI) staining. In MII, the nucleus will disappear and two spindles will appear. Magnification 40X

4.3.6.2. Determination of polyspermy following *in vitro* fertilization.

Following *in vitro* fertilization (procedure mentioned above in section 4.3.4), polyspermy was determined with DAPI staining. After 6 hr of fertilization, the fertilized oocytes were washed (3X) in washing buffer media. Oocytes were kept in a 50 µl droplet of protease (Trypsin; 5 mg/ml in washing buffer) for 2.5-3 min at 22 ºC until the width of zona pellucida was reduced to 50%. Oocytes were transferred into 50 µl droplet of blocking media using micropipette 2X (5 min each). The fertilized oocytes were transferred into 5 µl of DAPI droplets for 3X, and then the last droplet was mounted on the slide. The droplet was covered by cover slide supported by vaseline:parafilm on each corner of cover slip. Then, the glass slides were checked under epifluorescence microscope (Carl Zeiss, Axioskop 40, Germany: 40X). In this step of fertilization, the number of pronuclei were checked to recognize normal fertilization or polyspermy. Oocytes with one pronucleus indicated lack of fertilization. Two pronuclei in the oocyte represented normal fertilization and zygote development, and more than two pronuclei in oocytes indicated polyspermy (Figure 4.2). In this test, 121 pre-pubertal gilt oocytes divided amongst 3 replicates and 51 adult sow oocytes were used in total across 3 replicates.

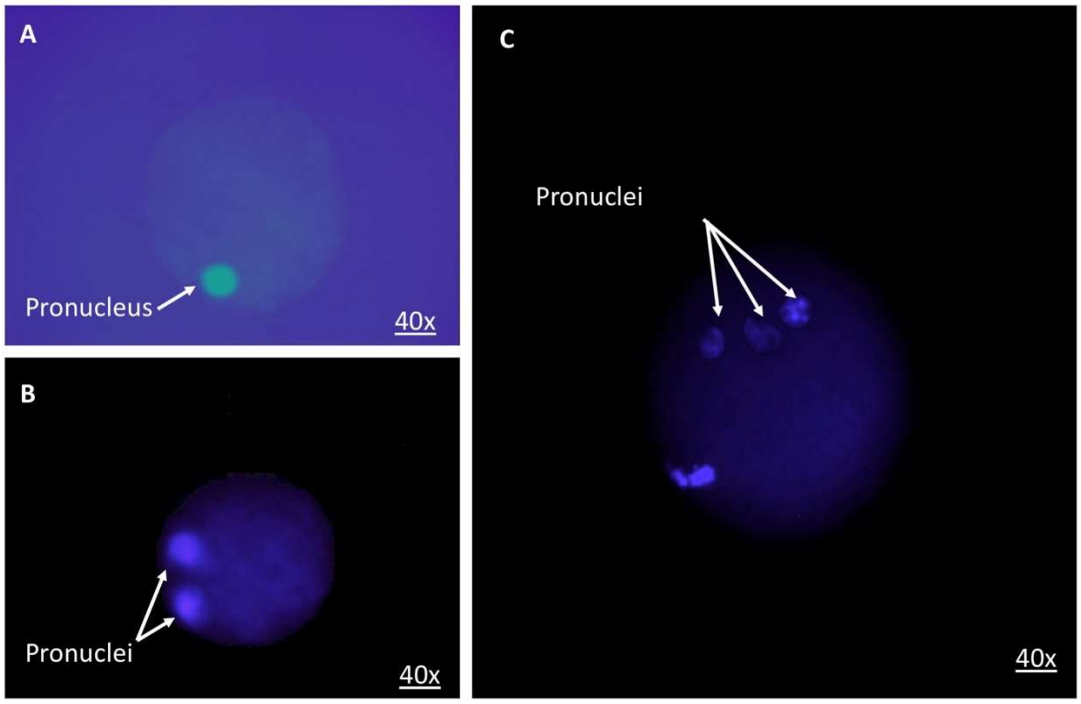

Figure 4.2: Classification of porcine oocytes for polyspermy using 4',6-diamidino-2-phenylindole (DAPI) staining. The magnification is 40X. In this figure, there is unfertilized oocyte with single pronucleus (A), normal fertilized oocyte with two pronuclei (B), and polyspermic oocyte with >2 pronuclei (C).

4.3.6.3. Determination of fertilization duration.

Following IVM in TCM-199 medium (5% CO_2, 5% O_2 and 90% N_2 in a humidified environment, and at 38.5˚C) for 44 hr as described in section 4.3.3, the oocytes were divided into two groups depending on fertilization duration (the time that sperm are exposed to the oocytes): a) control fertilized for 6 hr (n=122 oocytes divided into 3 replicates), and b) treatment (n=122 oocytes divided into 3 replicates) fertilized for 9hr. The fertilization medium was mTBM medium. After the fertilization period (6 hr or 9 hr), the fertilized oocytes were washed (3X) using NCSU23 culture media and cultured for 7 days as described in the same previous environment. Cleavage rate was evaluated in day 3.

4.3.7. Experiment 2: Evaluation of Mitochondrial and Cortical Granules Patterns in Different Maturation Media

The following maturation media were used in this experiment: Tissue Culture Medium (TCM)-199 (control, routinely used in our lab), North Carolina State University (NCSU) 23, modified Whitten's Medium (mWM), North Carolina State University (NCSU) 37 with glucose, and North Carolina State University (NCSU) 37 with pyruvate and lactate (Table 4.1).

4.3.7.1. Mitochondrial distribution

The best safe concentration established in chapter 2 of MTG (200 nM, section 3.3.1.1) was used to observe MT distribution. Pig oocytes stained with MitoTracker Green (MTG) were sorted into four categories: peripheral, semi-peripheral, diffused, and unclassified.

4.3.7.2. Cortical granules distribution

The best safe concentration figured out in chapter 2 of Peanut agglutinin (PNA; 625 nM, section 3.3.1.1) was used to determine CGs distribution. Pig oocytes stained with PNA were sorted into four categories: cortical, intermediate, peripheral, and unclassified.

4.3.8. Experiment 3: *In Vitro* Fertilization of Pig Oocytes Following Maturation in Different Media.

After maturation, IVF and embryo development were compared between maturation media. The matured oocytes from pre-pubertal gilts were divided into three main groups: Group 1, oocytes not stained and not processed for IVF (No stain & no IVF; negative control); Group 2, oocytes not stained but were processed for IVF following standard procedure (Standard IVF; positive control); Group 3, oocytes stained with PNA, subcategorized (for CG distribution patterns) and processed for IVF procedure. All oocytes were processed as described in sections 4.3.2 to 4.3.4.

4.3.9. Experiment 4: Determination of Polyspermy in Different Maturation Media

Pre-pubertal oocytes were used in this experiment. Following 6 hr post-fertilization, oocytes from no staining and stained with PNA were washed (3X) with a washing solution (PBS + 0.1% PVA). Presumptive fertilized oocytes were transferred into trypsin droplet (0.25% Trypsin with 0.02% EDTA in washing media) for 5 min to remove the accessory sperm around oocytes. After 5 min, the fertilized oocytes were washed (2X, 5 min each) in blocking media (2% BSA in PBS). Oocytes were fixed with 4% paraformaldehyde overnight. The next day, oocytes were washed (2X) in washing solution and transferred into a droplet (5 ul) of Vectashield with 4',6-diamidino-2-phenylindole (DAPI; section 4.3.6.2.). Number of pronuclei were counted using a fluorescent microscope (Carl Zeiss, Axioskop 40, Germany; 40X) to evaluate polyspermy. Two main groups were used in this experiment using PNA segregation: control group as a standard IVF and stained group. The stained group was divided into subgroups depending on the CGs' distribution (cortical, intermediate, peripheral, and unclassified patterns).

4.4. Statistical Analysis

Data from 3 or more replicates was compiled and analyzed using STATA 17.0 software (Copyright 1985-2021 StataCorp LLC, 4905 Lakeway Drive, College Station, Texas 77845 USA). The percentage of oocytes within each repetition was the outcome variable used for all

analyses. A zero-Skewness log transformation technique was applied before the statistical analysis to obtain a normal distribution. Differences across group (different media and/or different maturation stages) were assessing using a one-way ANOVA was after the transformation. If significant, a pairwise comparison with Tukey's multiple group adjustment was used to confirm differences among groups. A minimum 50 oocytes were used for this study based on the alpha = 0.05 and experiment power 90%.

4.5. Results

4.5.1. Experiment 1: Determination of Maturation and Polyspermic Fertilization (Preliminary Experiment)

4.5.1.1. Determination of maturation stage of pig oocytes

The maturation rate was higher ($P \leq 0.05$) in oocytes collected from adult sows (78% ± 2.00; 61/78) in comparison to oocytes from pre-pubertal gilts (56% ± 1.23; 94/168). On the other hand, the percentage of matured oocytes in MII stage were higher ($P \leq 0.05$) in both sources of oocytes than the other maturation stages (Table 4.2).

4.5.1.2. Determination of polyspermy following *in vitro* fertilization.

There was no significant difference in normal fertilization rate (49% ± 12.06; 59/121) in oocytes from pre-pubertal gilts as compared with oocytes from adult sows (55% ± 3.51; 28/51; Table 4.3), and the polyspermic fertilization rate did not differ between adult sows and pre-pubertal gilts (18% ± 1.73; 9/51; and 19% ± 2.31; 23/121; respectively). However, the polyspermic fertilization rate was less ($P \leq 0.05$) in comparison to normal fertilized zygotes in both pre-pubertal gilts and adult sow groups.

Table 4.2: Maturation rate (%) of oocytes after 44hr of maturation from pre-pubertal gilts and adult sow. Each value represents mean (%) ± SD.

Maturation status	Source of oocytes	
	Adult sows (**)	Pre-pubertal gilts (**)
GV NS	3 ± 0.74 a (x) (N=2) [4,4,0]	3 ± 0.34 a (w) (N=5) [4,2,3]
GVBD *	4 ± 2.11 b (x) (N=3) [4,4,4]	11 ± 0.60 a (x) (N=18) [11,9,12]
MI *	15 ± 1.28 b (y) (N=12) [15,19,12]	30 ± 0.60 a (y) (N=51) [29,34,29]
MII *	78 ± 2.00 b (z) (N=61) [77,73,85]	56 ± 1.23 a (z) (N=94) [57,55,56]
Total #	78	168

* Within a maturation status (row), values with different letters (a and b) are different (P≤0.05) between the source of oocytes.
** Within a source of oocytes (column), values with different letters (w-z) represent differences (P≤0.05) in maturation status.
NS represent non-significant difference in a row.
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.

Table 4.3: Fertilization and polyspermy rate (%) in oocytes from pre-pubertal gilts and adult sows' oocytes. Each value represents the mean (%) ±SD.

Fertilization status	Source of oocytes	
	Adult sows (*)	Pre-pubertal gilts (*)
Unfertilized NS	27±2.08 a (xy) (N=14) [28,27,27]	32±2.65 a (xy) (N=39) [33,26,45]
Normal fertilized zygotes NS	55±3.51 a (x) (N=28) [52,60,55]	49±12.06 a (x) (N=59) [47,57,32]
polyspermic fertilized oocytes NS	18±1.73 a (y) (N=9) [20,13,18]	19±2.31 a (y) (N=23) [20,17,23]
Total	51	121

* Within a source of oocytes (column), values with different letters (x and y) represent differences (P≤0.05) in fertilization status.
NS represent non-significant difference in a row.
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.

4.5.1.3. Determination of fertilization duration

To solve the low fertilization rate, we tried to change the fertilization duration. The standard duration of 6 hr with semen was used previously. In this case, we used 9 hr of fertilization duration with the semen before washing the zygotes from the semen. In this test, the cleavage rate decreased slightly from 51% ± 11.53 (45/88) for the 6 hr duration to 36% ± 24.27 (39/108) for the 9 hr duration using pre-pubertal gilts oocytes (Figure 4.3).

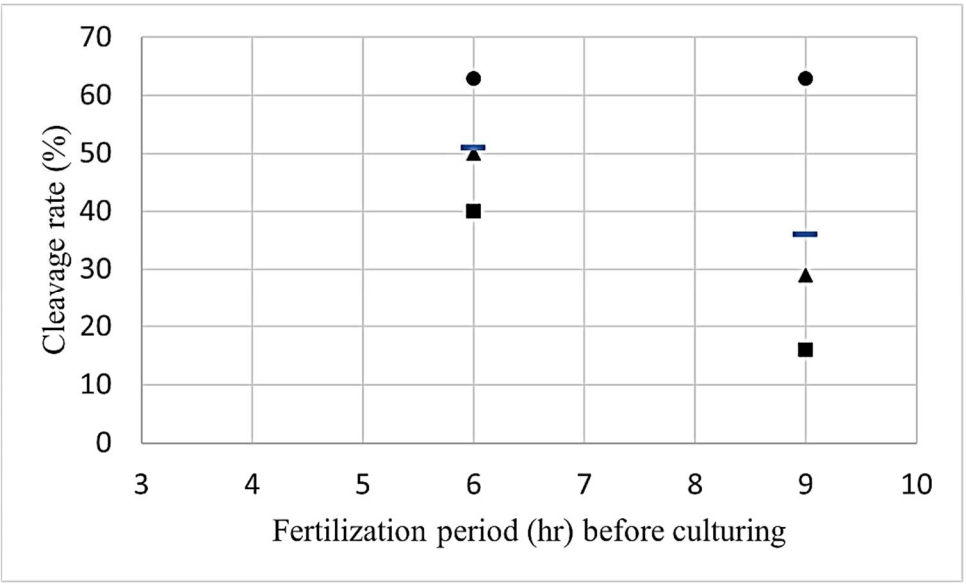

Figure 4.3: The cleavage rate (%) as a comparison between two washing periods after IVF using oocytes derived from pre-pubertal gilts. These periods are the periods what the sperms stand with oocytes for fertilization. The circles, squares, and triangles represent three replicates. The small lines represent the mean.

4.5.2. Experiment 2: Evaluation of Mitochondrial and Cortical Granules Patterns in Different Maturation Media

4.5.2.1. Mitochondrial distribution (Table 4.4)

Before maturation, the percentage of oocytes with peripheral pattern of MT was greater at 50% ± 9.45 (176/350) than the other patterns (P≤0.05). Among the media, the TCM media yielded the lowest percentage of oocytes with peripheral pattern after maturation (7% ± 0.58; 11/150; P≤0.05) in comparison to NCSU23 (15% ± 1.15; 22/150) and NCSU37G (12% ± 1.00; 18/150), and slightly lower than mWM (10% ± 1.00; 15/150) and NCSU37PL (11% ± 1.53; 17/150). On the other hand, the percentage with semi-peripheral pattern did not differ significantly among the media after and before maturation (P≥0.05). There was a high rate of the peripheral pattern (50% ± 9.45; 176/350) in comparison to diffused pattern (5% ± 1.00; 18/350) in the oocytes before maturation. The diffused pattern rate increased significantly (P≤0.05) from 5% ± 1.00 (18/350) before maturation to reach 55% ± 1.00 (82/150), 50% ± 1.00 (75/150), 40% ± 2.00 (60/150), 43% ± 0.58 (64/150), and 51% ± 1.53 (77/150) in TCM, mWM, NCSU23, NCSU37G and NCSU37PL, respectively. However, the TCM, mWM, and NCSU37PL had higher (P≤0.05) diffused pattern rates in comparison to NCSU23 and NCSU37G. The unclassified pattern rate did not differ among media and before maturation except in NCSU37PL that had only 10% ± 2.00 (15/150) unclassified oocytes. In all media, the percentage of oocytes with diffused pattern was 40-55% greater (P≤0.05) than the peripheral pattern (7-15%).

Table 4.4: Mitochondrial distribution patterns in oocytes from pre-pubertal gilts before and after 44 hr maturation in different media. Each value represents mean (%) ± SD.

		Mitochondrial pattern				Total oocytes
		Peripheral (**)	Semi-peripheral (NS)	Diffused (**)	Unclassified (**)	
Before maturation *		50 ± 9.45 a (z) (N=176) [48,50,53]	24 ± 3.06 b (x) (N=85) [25,23,25]	5 ± 1.00 c (z) (N=18) [5,6,5]	20 ± 2.89 b (x) (N=71) [22,22,18]	350
After maturation	TCM *	7 ± 0.58 a (y) (N=11) [6,8,6]	24 ± 1.00 c (x) (N=36) [26,22,24]	55 ± 1.00 d (x) (N=82) [52,56,54]	14 ± 0.00 b (x) (N=21) [14,14,14]	150
	mWM *	10 ± 1.00 a (xy) (N=15) [12,8,10]	22 ± 1.00 b (x) (N=33) [24,20,22]	50 ± 1.00 c (x) (N=75) [48,52,50]	18 ± 1.00 b (x) (N=27) [16,20,18]	150
	NCSU23 *	15 ± 1.15 a (x) (N=22) [16,12,16]	27 ± 1.15 b (x) (N=40) [28,28,24]	40 ± 2.00 c (y) (N=60) [36,44,40]	19 ± 1.15 a (x) (N=28) [20,16,20]	150
	NCSU37G *	12 ± 1.00 a (x) (N=18) [10,14,12]	29 ± 2.08 b (x) (N=43) [30,24,32]	43 ± 0.58 c (y) (N=64) [42,44,42]	17 ± 1.15 a (x) (N=25) [18,18,14]	150
	NCSU37PL *	11 ± 1.53 a (xy) (N=17) [8,12,14]	27 ± 1.53 b (x) (N=41) [30,24,28]	51 ± 1.53 c (x) (N=77) [48,54,52]	10 ± 2.00 a (y) (N=15) [14,10,6]	150

* Within a medium (row), values with different letters (a-d) are different (P≤0.05) among media.
** Within a pattern (column), values with different letters (x-z) represent differences (P≤0.05) in mitochondrial pattern.
NS represent non-significant difference in a column.
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.
Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate).

4.5.2.2. Cortical granules distribution (Table 4.5)

The cortical pattern rate did not differ across media after maturation i.e. 5% ± 2.31 (7/150), 8% ± 2.65 (12/150), 9% ± 0.58 (13/150), 6% ± 1.00 (9/150), and 5% ± 0.58 (8/150) for TCM, mWM, NCSU23, NCSU37G, and NCSU37PL, respectively. However, the cortical pattern rate in all media were significantly lower in comparison with before maturation (56% ± 10.00; 195/350). The intermediate pattern rate was significantly lower before maturation (20% ± 2.64; 69/350) in comparison to NCSU23 (34% ± 1.00; 51/150), NCSU37G (31% ± 0.58; 46/150), and NCSU37PL (30% ± 1.00; 45/150) after maturation. However, there was no significant differences in the intermediate pattern rate between the TCM (25% ± 2.52; 37/150) or mWM (26% ± 2.65; 39/150) after maturation and before maturation group (20% ± 2.64; 69/350). The intermediate pattern was significantly higher in NCSU23 (34% ± 1.00; 51/150) than TCM (25% ± 2.52; 37/150), but not among the other media. The peripheral pattern rate was highest in the TCM media (60% ± 3.00; 90/150) that differed ($P \leq 0.05$) compared to the mWM (46% ± 3.60; 69/150) and NCSU23 (42% ± 2.60; 63/150), but not NCSU37G (49% ± 1.53; 73/150) or NCSU37PL (49% ± 1.53; 74/150). However, the peripheral pattern rate after maturation was higher ($P \leq 0.05$) in all media than before maturation group (9% ± 1.00; 33/350). Within the unclassified group, a difference ($P \leq 0.05$) was observed between the TCM (10% ± 1.00; 15/150) and mWM (20% ± 1.00; 30/150) media, but there was no differences among other media or before maturation group (15% ± 2.52; 53/350). According to the differences in patterns within each media, all media after maturation had a lower rate of cortical pattern ($P \leq 0.05$; 5-9%) in comparison to peripheral pattern (42-60%). On the other hand, the before maturation group had high ($P \leq 0.05$) percentage of cortical pattern (56% ± 10.00; 195/350) in comparison to peripheral pattern (9% ± 1.00; 33/350).

Table 4.5: Cortical granules distribution patterns in oocytes from pre-pubertal gilts before and after 44 hr maturation in different media. Each value represents mean (%) ± SD.

		Cortical granules pattern				Total oocytes
		Cortical (**)	Intermediate (**)	Peripheral (**)	Unclassified (**)	
Before maturation *		56 ± 10.00 a (x) (N=195) [55,52,60]	20 ± 2.64 b (z) (N=69) [20,20,19]	9 ± 1.00 d (z) (N=33) [10,10,9]	15 ± 2.52 c (xy) (N=53) [15,16,14]	350
After maturation	TCM *	5 ± 2.31 a (y) (N=7) [2,10,2]	25 ± 2.52 b (xz) (N=37) [20,24,30]	60 ± 3.00 c (x) (N=90) [66,54,60]	10 ± 1.00 a (x) (N=15) [12,10,8]	150
	mWM *	8 ± 2.65 a (y) (N=12) [6,14,4]	26 ± 2.65 bc (xyz) (N=39) [22,24,32]	46 ± 3.60 c (y) (N=69) [50,40,44]	20 ± 1.00 b (y) (N=30) [18,22,20]	150
	NCSU23 *	9 ± 0.58 a (y) (N=13) [10,8,8]	34 ± 1.00 b (y) (N=51) [32,34,36]	42 ± 2.60 b (y) (N=63) [46,36,44]	15 ± 2.89 a (xy) (N=23) [12,22,12]	150
	NCSU37G *	6 ± 1.00 a (y) (N=9) [8,4,6]	31 ± 0.58 c (xy) (N=46) [30,32,30]	49 ± 1.53 d (xy) (N=73) [48,52,46]	15 ± 1.53 b (xy) (N=22) [14,12,18]	150
	NCSU37PL *	5 ± 0.58 a (y) (N=8) [6,4,6]	30 ± 1.00 c (xy) (N=45) [28,32,30]	49 ± 1.53 d (xy) (N=74) [50,52,46]	15 ± 1.53 b (xy) (N=22) [14,12,18]	150

* Within a medium (row), values with different letters (a-d) are different (P≤0.05) among media.
** Within a pattern (column), values with different letters (x-z) represent differences (P≤0.05) in mitochondrial pattern.
NS represent non-significant difference in a column.
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.
Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate).

4.5.3. Experiment 3: *In Vitro* Fertilization of Pig Oocytes Following Maturation in Different Media.

The cleavage rates did not differ across media for each of the CG patterns and control groups (standard IVF, no stain & no IVF, cortical, intermediate, and peripheral) as shown in Table 4.6. Within each media group, there were no significant difference between the standard IVF (64-70%) and the peripheral pattern groups (62-77%) in the cleavage rate, but both groups had higher rates ($P \leq 0.01$) than the other groups (15-18%, 24-50%, and 37-46% for no stain & no IVF, cortical, and intermediate patterns, respectively) except in NCSU23 media in which the cleavage rate differed ($P \leq 0.01$) only in no stain & no IVF group (18% ± 1.0; 16/75) compared to the standard IVF (70% ± 1.0; 105/150) and the peripheral pattern groups (77% ± 0.6; 83/108; Table 4.6).

In standard IVF (control) group, the morula rate (Table 4.7) was lower ($P \leq 0.01$) in the mWM (17% ± 1.2; 26/150) than both TCM (26% ± 2.6; 157/600) and NCSU37G (29% ± 1.5; 44/150). Also, there was a significant increase ($P \leq 0.01$) in morula rate in TCM (30% ± 2.3; 123/409) than both the mWM (21% ± 1.0; 21/102) and NCSU23 (20% ± 2.3; 22/108) within the peripheral group. In all other groups (no stain & no IVF, cortical, and intermediate), there were no differences in morula rate among media. In general, morula rates were higher ($P \leq 0.05$) in standard IVF (range 17-29%) and the peripheral (range 20-31%) groups than other patterns (0%, 0%, and 0-4% for no stain & no IVF, cortical, and intermediate groups, respectively).

The blastocyst rates (Table 4.8) did not differ among media across all oocyte pattern groups. However, in all media, the standard IVF (7-12%) and the peripheral pattern (10-15%) groups had higher ($P \leq 0.01$) blastocyst rates than all other patterns (no stain & no IVF, 0%; cortical, 0%; and intermediate, 0-1%). In addition, the TCM group had a higher blastocyst rate in peripheral pattern (15% ± 1.0; 60/409) than the standard IVF (10% ± 1.3; 62/600). As well, the blastocyte rate in NCSU37G medium was greater in the peripheral pattern (15% ± 1.0; 15/102) than the standard IVF (11% ± 0.6; 17/150).

Table 4.6: Cleavage rate (%) in control and sorted pig oocytes following maturation in different media and *in vitro* fertilization. Oocytes underwent standard IVF or received no-stain no-semen were considered as positive and parthenogenetic controls, respectively. Oocytes were sorted based on cortical granules distribution following PNA staining. Each value represents mean (%) ± SD.

	Media	Control group				Cortical granules group					
		Standard IVF		No stain & no IVF		Cortical		Intermediate		Peripheral	
		Total	(NS)	Total	(NS)	Total	(NS)	Total	(NS)	Total	(NS)
Cleavage rate (%)	TCM *	600	66 ±2.5 $^{a\,(x)}$ (N=397) [68,64,67]	258	17 ±0.9 $^{d\,(x)}$ (N=44) [16,19,15]	81	30 ±0.7 $^{c\,(x)}$ (N=23) [31,28,33]	117	46 ±1.4 $^{b\,(x)}$ (N=53) [41,45,51]	409	74 ±5.0 $^{a\,(x)}$ (N=305) [74,76,73]
	mWM *	150	65 ±0.6 $^{a\,(x)}$ (N=98) [66,64,66]	60	17 ±0.6 $^{c\,(x)}$ (N=10) [20,15,15]	18	28 ±0.6 $^{bc\,(x)}$ (N=5) [33,20,29]	30	37 ±0.6 $^{b\,(x)}$ (N=11) [36,36,38]	102	73 ±1.5 $^{a\,(x)}$ (N=74) [76,76,66]
	NCSU23 *	150	70 ±1.0 $^{a\,(x)}$ (N=105) [68,72,70]	75	18 ±1.0 $^{b\,(x)}$ (N=16) [20,12,16]	18	50 ±1.7 $^{ab\,(x)}$ (N=9) [40,29,83]	23	43 ±1.2 $^{ab\,(x)}$ (N=10) [40,33,57]	108	77 ±0.6 $^{a\,(x)}$ (N=83) [77,76,78]
	NCSU37G *	150	65 ±1.5 $^{a\,(x)}$ (N=97) [64,62,68]	75	15 ±1.5 $^{c\,(x)}$ (N=11) [16,20,8]	25	24 ±0.0 $^{c\,(x)}$ (N=6) [20,22,33]	37	46 ±1.2 $^{b\,(x)}$ (N=17) [42,47,50]	102	62 ±1.0 $^{a\,(x)}$ (N=63) [60,67,59]
	NCSU37PL *	150	64 ±1.0 $^{a\,(x)}$ (N=96) [66,62,64]	65	17 ±0.6 $^{c\,(x)}$ (N=11) [20,16,15]	21	29 ±0.0 $^{c\,(x)}$ (N=6) [33,25,29]	33	42 ±0.6 $^{b\,(x)}$ (N=14) [36,42,50]	97	70 ±2.1 $^{a\,(x)}$ (N=68) [76,65,70]

* Within a media (row), values with different letters (a-c) indicate differences (P≤0.01) among groups.
(NS) indicates no significant difference due to media, within a cortical granule group (column).
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.
Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate).

Table 4.7: Morula rate (%) in control and sorted pig oocytes following maturation in different media and *in vitro* fertilization. Oocytes underwent standard IVF or received no-stain no-semen were considered as positive and parthenogenetic controls, respectively. Oocytes were sorted based on cortical granules distribution following PNA staining. Each value represents mean (%) ± SD.

	Media	Control group				Cortical granules group					
		Standard IVF		No stain & no IVF		Cortical		Intermediate		Peripheral	
		Total	(**)	Total	(NS)	Total	(NS)	Total	(NS)	Total	(**)
Morula rate (%)	TCM *	600	26 ±2.6 a (x) (N=157) [24,26,29]	258	0 ±0.0 b (x) (N=0) [0,0,0]	81	0 ±0.0 b (x) (N=0) [0,0,0]	117	3 ±0.5 b (x) (N=4) [5,3,3]	409	30 ±2.3 a (x) (N=123) [29,30,32]
	mWM *	150	17 ±1.2 a (y) (N=26) [16,16,20]	60	0 ±0.0 b (x) (N=0) [0,0,0]	18	0 ±0.0 b (x) (N=0) [0,0,0]	30	0 ±0.0 b (x) (N=0) [0,0,0]	102	21 ±1.0 a (y) (N=21) [18,21,23]
	NCSU23 *	150	21 ±2.1 a (xy) (N=31) [16,24,22]	75	0 ±0.0 b (x) (N=0) [0,0,0]	18	0 ±0.0 b (x) (N=0) [0,0,0]	23	4 ±0.6 b (x) (N=1) [0,17,0]	108	20 ±2.3 a (y) (N=22) [17,27,17]
	NCSU37G *	150	29 ±1.5 a (x) (N=44) [30,26,32]	75	0 ±0.0 b (x) (N=0) [0,0,0]	25	0 ±0.0 b (x) (N=0) [0,0,0]	37	3 ±0.6 b (x) (N=1) [0,7,0]	102	31 ±1.5 a (xy) (N=32) [31,30,32]
	NCSU37PL *	150	23 ±1.5 a (xy) (N=35) [20,26,24]	65	0 ±0.0 b (x) (N=0) [0,0,0]	21	0 ±0.0 b (x) (N=0) [0,0,0]	33	3 ±0.6 b (x) (N=1) [0,8,0]	97	26 ±1.5 a (xy) (N=25) [21,29,27]

* Within a media (row), values with different letters (a and b) indicate differences (P≤0.01) among groups.
** Within a cortical granules pattern (column), different letters (x and y) represent differences (P≤0.01) due to media.
(NS) indicates no significant difference due to media, within a column.
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.
Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate).

Table 4.8: Blastocyst rate (%) in control and sorted pig oocytes following maturation in different media and *in vitro* fertilization. Oocytes underwent standard IVF or received no-stain no-semen were considered as positive and parthenogenetic controls, respectively. Oocytes were sorted based on cortical granules distribution following PNA staining. Each value represents mean (%) ± SD.

	Media	Control group				Cortical granules group					
		Standard IVF		No stain & no IVF		Cortical		Intermediate		Peripheral	
		Total	(NS)	Total	(NS)	Total	(NS)	Total	(NS)	Total	(NS)
Blastocyst rate (%)	TCM *	600	10 ±1.3 $^{b\,(x)}$ (N=62) [11,10,11]	258	0 ±0.0 $^{c\,(x)}$ (N=0) [0,0,0]	81	0 ±0.0 $^{c\,(x)}$ (N=0) [0,0,0]	117	1 ±0.3 $^{c\,(x)}$ (N=1) [2,0,0]	409	15 ±1.0 $^{a\,(x)}$ (N=60) [17,15,13]
	mWM *	150	8 ±1.7 $^{a\,(x)}$ (N=12) [10,10,4]	60	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	18	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	30	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	102	14 ±1.5 $^{a\,(x)}$ (N=14) [18,15,9]
	NCSU23 *	150	12 ±1.0 $^{a\,(x)}$ (N=18) [14,12,10]	75	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	18	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	23	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	108	13 ±0.6 $^{a\,(x)}$ (N=14) [14,14,11]
	NCSU37G *	150	11 ±0.6 $^{b\,(x)}$ (N=17) [12,10,12]	75	0 ±0.0 $^{c\,(x)}$ (N=0) [0,0,0]	25	0 ±0.0 $^{c\,(x)}$ (N=0) [0,0,0]	37	0 ±0.0 $^{c\,(x)}$ (N=0) [0,0,0]	102	15 ±1.0 $^{a\,(x)}$ (N=15) [14,13,16]
	NCSU37PL *	150	7 ±0.6 $^{a\,(x)}$ (N=11) [8,6,8]	65	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	21	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	33	0 ±0.0 $^{b\,(x)}$ (N=0) [0,0,0]	97	10 ±1.6 $^{a\,(x)}$ (N=10) [15,6,10]

* Within a media (row), values with different letters (a,b, and c) indicate differences (P≤0.01) among groups.
(NS) indicates no significant difference due to media, within a cortical granule group (column).
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.
Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate).

4.5.4. Experiment 4: Determination of Fertilization Status in Different Maturation Media

The rate of unfertilized oocyte (characterized by one pronucleus) was affected by media and was higher (34% ± 5.00; 128/381, P≤0.01) in NCSU37PL than other media (TCM, 22% ± 4.17; 297/1357; mWM, 20% ± 3.52; 68/338; NCSU23, 19% ± 3.00; 62/330; and NCSU37G, 15% ± 2.39; 53/345; Table 4.9). There were no differences in the percentage of zygotes with two pronuclei and more than two pronuclei among the media. In addition, the rate of fertilized oocytes (two pronuclei) was higher than unfertilized oocytes (one pronucleus) in all media groups TCM (52% ± 10.94; 704/1357 vs. 22% ± 4.17; 297/1357), mWM (51% ± 11.00; 173/338 vs. 20% ± 3.52; 68/338), NCSU23 (52% ± 9.98; 173/330 vs. 19% ± 3.00; 62/330) and NCSU37G (52% ± 11.16; 179/345 vs. 15% ± 2.39; 53/345) except NCSU37PL media. However, in NCSU37PL the rate of unfertilized and normal fertilized oocytes did not differ significantly. The rate of normal and polyspermic zygotes were not significantly different in TCM, mWM and NCSU37G. However, the rate of polyspermic zygotes was lower than normal zygotes in NCSU23 and NCSU37PL.

Table 4.9: The effect of media and different fertilization status in pig oocytes and zygotes, following *in vitro* maturation and fertilization. Each value represents mean (%) ± SD.

Media	Grand total	Fertilization status		
		Unfertilized (**)	Normal (NS)	Polyspermic (NS)
TCM *	1357	22 ±4.17 b (y) (N=297) [23,21,22]	52 ±10.94 a (x) (N=704) [50,54,53]	26 ±3.96 ab (x) (N=356) [27,26,25]
mWM *	338	20 ±3.52 b (y) (N=68) [18,20,22]	51 ±11.00 a (x) (N=173) [50,51,52]	29 ±4.61 ab (x) (N=97) [31,29,26]
NCSU23 *	330	19 ±3.00 b (y) (N=62) [13,17,26]	52 ±9.98 a (x) (N=173) [54,55,49]	29 ±5.30 b (x) (N=95) [33,29,25]
NCSU37G *	345	15 ±2.39 b (y) (N=53) [14,15,17]	52 ±11.16 a (x) (N=179) [53,51,52]	33 ±5.01 a (x) (N=113) [33,34,31]
NCSU37PL *	381	34 ±5.00 a (x) (N=128) [36,34,31]	44 ±8.74 a (x) (N=168) [45,41,45]	22 ±3.20 b (x) (N=85) [19,25,24]

* Within a media (row), values with different letters (a and b) are different (P≤0.05) between fertilization status

** Within a fertilization status (column), different letters (x and y) differences due to media (P≤0.01).

(NS) indicates to no significant difference due to media.

N represents number of oocytes used.

The numbers between square brackets represent the percentage of each replicate.

Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University-37 with pyruvate and lactate)

The rate of unfertilized oocytes (characterized by one pronucleus) differed in standard IVF and peripheral CGs groups among media (Table 4.10). In standard IVF (control), the percentage of unfertilized oocytes was higher in NCSU37PL (33% ± 1.5; 50/150) than in NCSU37G (15% ± 1.5; 23/150) and NCSU23 (17% ± 3.2; 25/150) ($P \leq 0.05$). The percentage of unfertilized oocytes in peripheral CGs group was higher in NCSU37PL (23% ± 1.5; 22/96) than NCSU37G (11% ± 1.5; 10/93; $P \leq 0.05$).

Within the TCM media, the rate of unfertilized oocytes was lower in peripheral group (17% ± 1.7; 63/372) than in the unclassified pattern (31% ± 2.5; 30/96, $P \leq 0.01$). There were no significant differences among the CG pattern groups within the mWM or NCSU23 media. In NCSU37G, the unfertilized oocyte rate was lower ($P \leq 0.01$) in the peripheral (11% ± 1.5; 10/93) and intermediate (13% ± 0.0; 6/47) patterns than in the unclassified (26% ± 0.6; 5/19) and cortical patterns (25% ± 0.0; 9/36); and the unfertilized oocyte rate was lower in standard IVF (15% ± 1.5; 23/150) than in the unclassified pattern (26% ± 0.6; 5/19). However, in NCSU37PL, the unfertilized oocyte rate was lower ($P \leq 0.01$) in the peripheral pattern (23% ± 1.5; 22/96) than all other CGs groups (standard IVF, 33% ± 1.5; 50/150; unclassified, 42% ± 5.3; 18/43; cortical, 51% ± 3.1; 20/39; and intermediate, 34% ± 2.7; 18/53). Also, the rate of unfertilized oocytes was lower ($P \leq 0.01$) in standard IVF (33% ± 1.5; 50/150) and intermediate (34% ± 2.7; 18/53) patterns than in the cortical pattern (51% ± 3.1; 20/39).

The rate of presumptive zygotes with normal fertilization (characterized by two pronuclei; Table 4.11) was higher ($P \leq 0.05$) in the TCM media (53% ± 2.5; 326/610) than the NCSU37PL (45% ± 2.1; 67/150) within the standard IVF. However, there were no significant differences among all media within the unclassified and intermediate CGs patterns. In peripheral pattern, the percentage of zygotes with normal fertilization were lower ($P \leq 0.05$) in NCSU37PL (58% ±4.5; 65/96) in comparison with TCM (71% ± 2.8; 263/372), mWM (71% ± 2.1; 70/99), NCSU23 (73% ± 2.1; 61/84); and NCSU37G (75% ± 2.5; 70/93). In all media, the peripheral pattern had higher ($P \leq 0.01$) rate of normal zygotes (with two pronuclei) than standard IVF, cortical, intermediate, and unclassified. On the other hand, the cortical pattern group had lowest percentage of normal zygotes than all other pattern groups ($P \leq 0.01$).

The rate of zygotes with polyspermic fertilization (characterized by more than two pronuclei) did not differ among media within the standard IVF, intermediate pattern, or unclassified pattern (Table 4.12). However, within the cortical pattern, the rate of zygotes with

polyspermy was lowest (P≤0.05) in the NCSU37PL (33% ± 1.5; 13/39) compared to mWM (68% ± 1.5; 19/28) and NCSU37G (67% ± 1.0; 24/36). In peripheral pattern, the percentage of polyspermic zygotes was higher (P≤0.05) in NCSU37PL (19% ± 1.0; 18/96) in comparison to TCM (12% ± 0.9; 46/372) and mWM (10% ± 1.2; 10/99). Overall, within each media, except NCSU37PL, there was lower (P≤0.05) rate of polyspermic fertilization in the peripheral pattern in comparison to all other patterns (standard IVF, cortical, intermediate, and unclassified).

A summary of some advantages and disadvantages of each media is shown in table 4.13.

Table 4.10: Effect of media and cortical granules patterns on unfertilized pig oocytes (characterized by one pronucleus). Each value represents mean (%) ± SD.

Unfertilized oocytes (%)	Media	Cortical granule pattern									
		Standard IVF		Unclassified		Cortical		Intermediate		Peripheral	
		Total	(**)	Total	(NS)	Total	(NS)	Total	(NS)	Total	(**)
	TCM *	610	21 ±4.9 ab (xy) (N=131) [22,20,23]]	96	31 ±2.5 a (x) (N=30) [35,27,30]	125	30 ±2.2 ab (x) (N=37) [29,30,31]	154	23 ±1.5 ab (x) (N=36) [21,27,22]	372	17 ±0.00 b (xy) (N=63) [17,17,17]
	mWM NS	150	20 ±2.0 a (xy) (N=30) [20,16,24]	20	30 ±0.0 a (x) (N=6) [25,29,40]	28	21 ±0.0 a (x) (N=6) [18,25,22]	41	17 ±1.5 a (x) (N=7) [7,17,27]	99	19 ±2.1 a (xy) (N=19) [19,24,13]
	NCSU23 NS	150	17 ±3.2 a (y) (N=25) [12,14,24]	20	20 ±0.6 a (x) (N=4) [20,25,14]	33	30 ±1.2 a (x) (N=10) [20,33,36]	43	26 ±2.1 a (x) (N=11) [17,19,40]	84	14 ±2.7 a (xy) (N=12) [9,10,22]
	NCSU37G *	150	15 ±1.5 ab (y) (N=23) [12,16,18]	19	26 ±0.6 c (x) (N=5) [25,33,22]	36	25 ±0.0 bc (x) (N=9) [27,23,25]	47	13 ±0.0 a (x) (N=6) [14,13,11]	93	11 ±1.5 a (y) (N=10) [11,7,14]
	NCSU37PL *	150	33 ±1.5 b (x) (N=50) [34,36,30]	43	42 ±5.3 ab (x) (N=18) [43,40,40]	39	51 ±3.1 a (x) (N=20) [53,44,55]	53	34 ±2.7 b (x) (N=18) [36,36,29]	96	23 ±1.5 c (x) (N=22) [24,24,21]

* Within a media (row), values with different letters (a-c) are different (P≤0.05) between the cortical granules pattern.
** Within a cortical granule pattern (column), different letters (x and y) represent differences due to media (P≤0.05).
(NS) indicates that there is no significant difference due to media.N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.
Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate)

Table 4.11: Effect of media and cortical granules patterns on normal fertilized zygotes (characterized by 2 pronuclei). Each value represents mean (%) ± SD.

	Media	Cortical granules pattern									
		Standard IVF		Unclassified		Cortical		Intermediate		Peripheral	
		Total	(**)	Total	(NS)	Total	(**)	Total	(NS)	Total	(**)
Normal fertilized zygote (%)	TCM *	610	53 ±2.5 $^{b\,(x)}$ (N=326) [51,56,54]	96	36 ±2.6 $^{c\,(x)}$ (N=35) [37,35,37]	125	15 ±0.7 $^{d\,(xy)}$ (N=19) [15,20,11]	154	40 ±1.6 $^{c\,(x)}$ (N=61) [39,40,39]	372	71 ±2.8 $^{a\,(x)}$ (N=263) [71,71,70]
	mWM *	150	51 ±1.5 $^{b\,(xy)}$ (N=76) [50,54,48]	20	35 ±0.6 $^{c\,(x)}$ (N=7) [38,29,40]	28	11 ±0.0 $^{d\,(y)}$ (N=3) [9,13,11]	41	41 ±0.6 $^{bc\,(x)}$ (N=17) [43,42,40]	99	71 ±2.1 $^{a\,(x)}$ (N=70) [69,64,80]
	NCSU23 *	150	51 ±1.2 $^{b\,(xy)}$ (N=77) [50,54,50]	20	50 ±0.6 $^{b\,(x)}$ (N=10) [60,50,43]	33	21 ±0.6 $^{c\,(x)}$ (N=7) [20,25,18]	43	42 ±1.0 $^{b\,(x)}$ (N=18) [50,44,33]	84	73 ±2.1 $^{a\,(x)}$ (N=61) [78,76,66]
	NCSU37G *	150	53 ±0.6 $^{b\,(xy)}$ (N=79) [52,52,54]	19	37 ±0.6 $^{b\,(x)}$ (N=7) [50,33,33]	36	8 ±0.0 $^{c\,(y)}$ (N=3) [9,8,8]	47	43 ±0.6 $^{b\,(x)}$ (N=20) [50,40,39]	93	75 ±2.5 $^{a\,(x)}$ (N=70) [75,77,74]
	NCSU37PL *	150	45 ±2.1 $^{b\,(y)}$ (N=67) [46,40,48]	43	42 ±6.1 $^{b\,(x)}$ (N=18) [46,40,30]	39	15 ±1.0 $^{c\,(xy)}$ (N=6) [16,11,18]	53	40 ±3.0 $^{b\,(x)}$ (N=21) [40,36,41]	96	58 ±4.5 $^{a\,(y)}$ (N=65) [61,56,58]

* Within a media (row), values with different letters (a-c) are different (P≤0.05) between the cortical granules pattern.
** Within a cortical granule pattern (column), different letters (x and y) represent differences due to media (P≤0.05).
(NS) indicates that there is no significant difference due to media.
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.
Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate)

Table 4.12: Effect of media and cortical granules patterns on polyspermic fertilized oocytes (characterized by >2 pronuclei). Each value represents mean (%) ± SD.

Polyspermic oocytes (%)	Media	Cortical granules pattern									
		Standard IVF		Unclassified		Cortical		Intermediate		Peripheral	
		Total	(NS)	Total	(NS)	Total	(**)	Total	(NS)	Total	(**)
	TCM *	610	25 ±2.1 b (x) (N=153) [27,25,24]	96	32 ±1.4 b (x) (N=31) [28,38,33]	125	55 ±2.1 a (xy) (N=69) [56,55,57]	154	37 ±1.7 b (x) (N=57) [39,33,39]	372	12 ±0.9 c (y) (N=46) [11,13,13]
	mWM *	150	29 ±0.6 b (x) (N=44) [30,30,28]	20	35 ±1.2 b (x) (N=7) [38,43,20]	28	68 ±1.5 a (x) (N=19) [73,63,67]	41	41 ±1.2 ab (x) (N=17) [50,42,33]	99	10 ±1.2 c (y) (N=10) [11,12,7]
	NCSU23 *	150	32 ±3.0 a (x) (N=48) [38,32,26]	20	30 ±1.0 a (x) (N=6) [20,25,43]	33	48 ±0.6 a (xy) (N=16) [60,42,45]	43	33 ±1.2 a (x) (N=14) [33,38,27]	84	13 ±0.6 b (xy) (N=11) [13,14,13]
	NCSU37G *	150	32 ±2.0 b (x) (N=48) [36,32,28]	19	37 ±1.5 b (x) (N=7) [25,33,44]	36	67 ±1.0 a (x) (N=24) [64,69,67]	47	45 ±2.0 ab (x) (N=21) [36,47,50]	93	14 ±0.6 c (xy) (N=13) [14,17,11]
	NCSU37PL NS	150	22 ±1.00 a (x) (N=33) [20,24,22]	43	16 ±1.2 a (x) (N=7) [11,20,30]	39	33 ±1.5 a (y) (N=13) [32,44,27]	53	26 ±1.5 a (x) (N=14) [24,27,29]	96	19 ±1.0 a (x) (N=18) [16,20,21]

* Within a media (row), values with different letters (a-c) are different (P≤0.05) between the cortical granules pattern.
** Within a cortical granule pattern (column), different letters (x and y) represent differences due to media (P≤0.05).
NS indicates that there is no significant difference due to pattern.
(NS) indicates that there is no significant difference due to media.
N represents number of oocytes used.
The numbers between square brackets represent the percentage of each replicate.
Abbreviations of media: TCM (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate)

Table 4.13: some advantages and disadvantages using different media with mature sorted oocytes (MT diffused or CGs peripheral).

Media	MT distribution *	CG distribution *	Cleavage ^{NS}	Morula *	Blastocyst ^{NS}	Unfertilized oocytes *	Normal fertilized zygotes *	Polyspermy rate *
TCM-199	High	High	NS	High	NS	NS	High	Low
mWM	High	Low	NS	Low	NS	NS	High	Low
NCSU23	Low	Low	NS	Low	NS	NS	High	Low
NCSU37G	Low	Low	NS	High	NS	Low	High	Low
NCSU37PL	High	Low	NS	Low	NS	High	Low	High

* High (significant increase percentage of the character using the media), Low (significant decrease percentage of the character using media), NS: No significant effect.
Abbreviations: MT (Mitochondria), CG (Cortical granules), TCM-199 (Tissue Culture Medium 199), NCSU23 (North Carolina State University 23), mWM (modified Whitten's Medium), NCSU37G (North Carolina State University 37 with glucose), and NCSU37PL (North Carolina State University 37 with pyruvate and lactate).

4.6. Discussion

This study was designed to evaluate the effects of maturation media, MT distribution, CGs distribution for IVEP and the polyspermy rate in pigs. In the preliminary experiments, we attempted to identify problems in pig IVEP processing leading to poor embryo development. To determine the maturation of pre-pubertal or adult sows oocytes, anti Lamin/DAPI staining was used to determine the maturation status of pig oocyte in comparison with a previous results from our lab (AL-SHANOON, 2016). In adult sows, 78% ± 2.00 (61/78) of oocytes reached maturation (MII) in comparison to 56% ± 1.23 (94/168) of pre-pubertal oocytes. This difference was due to reproductive maturity, and oocytes from adult sows usually have better maturation competence than the young gilts as indicated by earlier research demonstrating more oocytes collected from adults reached the MII stage than young gilts (MARCHAL et al., 2001).

There was no significant difference in the polyspermy rate between the adult (18% ± 1.73; 9/51) and pre-pubertal oocytes (19% ± 2.31; 23/121). This similarity may be related to the fact that both groups had a similar maturation competency at the time of oocytes harvesting. Unfortunately, due to the lack of availability of adult sows for slaughtering, oocytes from pre-pubertal gilts were the main source for the subsequent experiments.

To solve the low percentage of fertilization, a preliminary experiment was undertaken by delaying the post-fertilization culture from 6 hr (as a standard period) to 9 hr. The cleavage rate in this preliminary experiment decreased from 51% ± 11.53 (45/88) in 6 hr to 36% ± 24.27 (39/108) after 9 hr fertilization time. The increase in fertilization time may improve the chances of sperm penetration into the oocytes, but not embryos development. Therefore, we used the normal time (6 hr) in the subsequent experiments because the longer time (9 hr) may increase the polyspermy rate and then decrease the cleavage rate (YI et al., 2004, PARK et al., 2003). The difference between our results at 6 hr (51% ± 11.53; 45/88) and the previous result from our lab (7%) may be due to logistic problems associated with earlier experiment that was conducted during the cold months of December and January (AL-SHANOON, 2016). A possible reason for the difference in the results between the pre-pubertal and sow oocytes reported herein compared to the previous study may be the techniques used. According to this difference in technique, we developed some procedures to reduce the environmental effects affecting the ovaries and oocytes collected. Instead of using a thermo container alone to transport the ovaries from the

slaughterhouse, we placed the ovaries in a plastic bag and covered with thick cotton in addition to using the thermo container to keep ovaries temperature at 37 °C. Another development was adapting ovaries at 24 °C with glucose solution instead of 37 °C to be more certain that the temperature of the ovaries dropped slightly without any temperature shock.

Using MTG, our study established that about 50% ± 9.45 (176/350) of the oocytes did not initiate a translocation of MT from peripheral to diffuse patterns, an indicator of oocytes' maturation (PAWLAK et al., 2016a). Among the remaining oocytes 24% ± 3.06 (85/350) initiated and 5% ± 1.00 (18/350) completed the MT translocation associated with maturation. Collected oocytes from ovaries usually are in different stages of initiating maturation at the harvesting time (SUN et al., 2001). After 44 hr of maturation, a significant portion of the oocytes' MT translocated to a diffuse pattern. This translocation correlated with the mature oocytes (55% ± 1.00; 82/150; 50% ± 1.00; 75/150; 40% ± 2.00; 60/150; 43% ± 0.58; 64/150; and 51% ± 1.53; 77/150 in TCM, mWM, NCSU23, NCSU37G, and NCSU37PL, respectively). This translocation depends on the stage of maturation. The MT is translocated from cortical area of the cytoplasm in immature or maturing oocytes to the central region of the cytoplasm in the matured oocytes (PAWLAK et al., 2016a). In our study, the diffused pattern was significantly higher in TCM, mWM, and NCSU37PL media. All these media contain the pyruvate in their composition. Pyruvate is possibly a metabolic requirement in MT during maturation. The MT consume adenosine triphosphate (ATP) faster in the presence of pyruvate (UDAGAWA and ISHIHARA, 2020). MT are translocated using oocytes' microtubules through motor proteins in the ATP consumption sites (YAMOCHI et al., 2016). Since the unclassified pattern occurred by the poor signal of MTG that could not be recognized under the stereo wide-field microscope, this pattern is not important in our study. The fluorescent signals from peripheral, semi-peripheral and diffused patterns were clear and distinguishable.

Using PNA to identify the CGs distribution, our results revealed that 56% ± 10.00 (195/350) of oocytes expressed cortical pattern at the time of collection, indicating 44% (155/350) of oocytes had already initiated the maturation. Once again, the unclassified pattern was mainly due to the poor signal of PNA under the stereo wide-field microscope. Therefore, this pattern is not important in our study as the other patterns. On the other hand, only 9% ± 1.00 (33/350) of oocytes had a peripheral pattern of CG at the time of collection which increased to 42-60% after maturation in the different media evaluated. CG translocation may be affected by

calcium supplementation in maturation media. Calcium activates the Transient Receptor Potential cation channel (TRPV3) (LEE et al., 2016) and/or Store-Operated Calcium Entry (SOCE) (XU and YANG, 2017) and subsequently controls actin filaments. Actin filaments are responsible for movement of CGs during their translocation (LIU, 2011, CHEESEMAN et al., 2016b). TCM media had a greater rate of peripheral pattern of CGs (60% ± 3.00; 90/150) than the other media (42-49%) or before maturation group (9% ± 1.00; 33/350) likely because it contains more calcium than other media (1.8 mM/L vs. 1.7 mM/L; as we prepared these media) since the calcium is an activating factor of CGs translocation (FERREIRA et al., 2009). For this reason, all media had significant translocation from cortical pattern (5-9%) to peripheral pattern (42-60%) after maturation. Due to limitations in time and resources, the rest of this study was based on CGs distribution ignoring the effects of MT distribution.

In IVEP experiment, oocytes were harvested from slaughtered pre-pubertal gilts without knowledge of their estrous cycle stage at the time of slaughtering, so the harvested oocytes might be under several hormonal influences of the source ovaries. There was no significant difference in cleavage, morula, or blastocyst rates between the standard IVF and the peripheral pattern within all media except for the blastocyst rate in TCM and NCSU37G that was significantly higher in the peripheral pattern group. *In vitro* fertilization of pig oocytes has always had a low overall success rate (ROMAR et al., 2019, ROMAR et al., 2016). However, there were significant increases in these patterns than the other groups (no stain & no IVF, cortical, and intermediate) among all media except in the cleavage rate of NCSU23. This lack of significance across media may be caused by the high variation through the replicates within the cortical pattern (40%, 29%, and 83%) and intermediate pattern (40%, 33%, and 57%). It is well recognised that grouping embryos improved their development due to embryo-embryo interactions (PARIA and DEY, 1990). If our sorting technique segregates the oocytes of the same cytoplasmic maturation stage, the embryos developed may exhibit the same autocrine/paracrine factors needed to mature and develop into embryos. The autocrine/paracrine factors usually are the same at equivalent maturation stages (WYDOOGHE, 2017, MACHTINGER et al., 2016). We may therefore assume that grouping oocytes with a similar CGs patterns generates a unique environment, which can impact the development of embryos collectively. To detect cytoplasmic maturity, just one marker (PNA) was applied. In future studies, additional markers may be required for the segregation of fully developed oocytes.

Lastly, we cannot exclude the possibility that the healthy embryos may release negative paracrine substances that impair the development of other embryos (WYDOOGHE, 2017).

There was no significant difference among the media used in cleavage and blastocyst rates, but the morula rate was higher in TCM for both the standard IVF and the peripheral patterns than the other media, and slightly differed than NCSU37G. This may be related to the higher glucose concentration in the TCM than the other media. There is a positive correlation between the glucose uptake and the embryo development, especially in morula and blastocyst stages (JONES et al., 2001). In general, the embryo development was not affected by media type. Currently, all media used in our study are available commercially for use in pig oocytes maturation (FOWLER et al., 2018). For this reason, it is possible that the media used in our study have the same requirements for maturing oocytes.

Polyspermy is one of the common adverse event in porcine embryo development (GRUPEN, 2014). Various studies have shown low polyspermy rates related to sperm concentration management (COY et al., 1993b), addition of oviductal or seminal fluids (BATISTA et al., 2016), development of a novel maturation medium (ROMAR et al., 2016), or protein addition (SAAVEDRA et al., 2014). In this experiment, the polyspermic fertilization rate was slightly lower in NCSU37PL (22% ± 3.20; 85/381) and TCM (26% ± 3.96; 356/1357) than the other media (29-33%). It seems high and low polyspermy rates are attributed the presence or absence of peripheral CGs distribution. Pyruvate prevents early exocytosis of CGs and decreases the zona pellucida digestion time resulting in enhanced fertilization through the inhibition of oocyte aging (LIU et al., 2009). In all media used in this study, the sorted (matured) oocytes and the standard IVF treatment had superior result in normal fertilization than the other immature oocyte groups. The CGs in the cortex regions beneath the plasma membrane (peripheral pattern) are responsible for the release of calcium-dependent contents that modify the zona pellucida and block protease activity to minimize the polyspermy (YU et al., 2021).

Our experiments had only 3 replicates and tested several media, so the results should not be considered definitive. They might suggest trends that could be followed up with additional experiments with more replicates and fewer media groups to confirm. Also, all of the ANOVA tests done within a table were inter-related because the percentages in one cell affect multiple ANOVAs. This is a weakness of the statistical analyses. If there were many more replicates, a more sophisticated statistical analyses could be done. However, the variations in almost all

groups were very low that gave a good indicator of the statistical validity except in the cleavage rate of cortical and intermediate patterns within the NCSU23 media.

4.7. Conclusions

The maturation stage (MII) in adult sow was significantly higher than the pre-pubertal gilts, but there was no difference in polyspermic fertilization between them. Using 6 hr of fertilization time was better than using 9 hr with regards to the cleavage rate. All media containing pyruvate supplement (TCM, mWM, and NCSU37PL) had more mature oocytes based on the diffused pattern of MT and peripheral pattern of CGs. Yet, all media used in our study were equivalent in IVEP outcome at the blastocyst stage. However, the sorted oocytes with matured pattern (peripheral) and the standard IVF had greater cleavage, morulae, and blastocysts rates than immature (cortical) or maturing (intermediate) groups. In our study, polyspermic fertilization was investigated in oocytes sorted according to the distribution or the pattern of CG, giving the benefit of supporting CG exocytosis depending on the maturation status. This study found that polyspermic fertilization is most frequent with immature oocytes; however, in sorted (mature) oocytes, the quantity of sperm entering oocytes may be reduced by sorting on the basis of GCs pattern regardless of media used. We propose that mature oocytes be sorted to reduce the negative impact of polyspermic fertilization on monospermic zygotes during embryo development especially when using a media provided with pyruvate.

CHAPTER 5: VITRIFICATION AND TRANSFER OF PIG EMBRYOS ORIGINATED FROM SORTED OOCYTES

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Relationship of this study to the dissertation

In chapter 5, the ability of the sorted oocytes to form embryos, following *in vitro* fertilization, vitrification and embryo transfer was determined. Mitochondrial and cortical granules distributions in vitrified-sorted oocytes were evaluated (as in chapter 2). The viability, quality and developmental competence of non-vitrified and vitrified pig embryos was determined. In the end, the morula-stage embryos were transferred into pre-pubertal gilts. We hypothesized that pig embryos produced in *in vitro* from mitochondrial and cortical granules-sorted oocytes survive cryopreservation better and establish pregnancy following in vivo transfer.

Authors' Contributions

Hamza Al-Maamory: Perform the study, experimental design, write the manuscript.
Carl Lessard: Participate in the experimental design, supervising and critical revision the manuscript.

5.1. Abstract

Vitrification of embryos is one of the approaches for long-term conservation of pig genetic resources. Preserving endangered breeds can be done by increasing and storing the number of pure breed embryos using *in vitro* fertilization (IVF), vitrification and transfer of embryos. We hypothesized that *in vitro* produced pig embryos from mature sorted oocytes, based on mitochondria (MT) and cortical granules (CG), survive cryopreservation better to gain pregnancy after *in vivo* transfer, than embryos from non-sorted oocytes. The objectives of this study were to evaluate: 1) the viability of vitrified pig embryos derived from sorted oocytes; and 2) the pregnancy rate following the embryo transfer of non-vitrified (fresh) and vitrified embryos in pre-pubertal gilts. In Experiment 1, oocytes were matured *in vitro*, denuded cumulus cells and stained with MitoTracker Green (MTG) or FITC-labelled Peanut Agglutinin (PNA). Matured oocytes based on MTG and PNA staining, were sorted for cytoplasmic maturation, separately fertilized *in vitro* following standard procedure. Embryos (morulae) from diffused MT- and peripheral CG-sorted oocytes underwent vitrification and warming procedures. Morulae were evaluated for viability, quality and development to blastocyst stage. The non-sorted and non-vitrified embryos served as control. In Experiment 2, the non-vitrified and vitrified embryos were transferred in pre-pubertal gilts, and ultrasound examination and necropsy were conducted to confirm the pregnancy. There were significant differences ($P<0.05$) in viability, embryo (grade 1) quality, and blastocyst formation rate stage between non-vitrified group (98-100%, 81-87%, and 12-16%, respectively) and vitrified group (39-43%, 2-4%, 0%, respectively). There were no significant differences between the sorted and non-sorted oocytes, within vitrified and non-vitrified groups. No pregnancy was achieved following the transfer of vitrified and non-vitrified embryos; however, many corpora lutea on ovary in one of the treatment group pre-pubertal gilt. In conclusion, vitrification-warming procedures adversely affected the embryos survival, quality, and formation of blastocysts. The transfer of embryos into recipient pigs did not succeed.

5.2. Introduction

Vitrification is a cryopreservation procedure in which extra- and intra-cellular ice crystal formation is minimized by increasing the viscosity of the cryoprotectnat solution and using high cooling velocity (YAVIN and ARAV, 2007). Vitrification avoids damage to cells' mitochondria, DNA, plasma membrane, and nucleus due to intracellular and extracellular ice formation in slow-freezing cryopreservation procedure (DOBRINSKY and JOHNSON, 1994). The success of vitrification depends on many factors: cooling rate, the concentration and behaviour of cryoprotectants, and other additives to the media, and sample volume (SARAGUSTY and ARAV, 2011). Increasing cooling rate from 20,000 ºC/minute to 135,000 ºC/minute yielded 33.9% in survival rate of porcine blastocysts after vitirificaiton and warming (BEEBE et al., 2005). The concentration of cryoprotectants affects the survival rate of porcine blastocyst. The vitrification solution containing 16.5% (v/v) of dimethylsulfoxide and ethylene glycol each yielded 67% survival rate of blastocysts, but it was not significantly higher than 57% survival rate when solution containing 20% (v/v) of dimethylsulfoxide and ethylene glycol each was used (BERTHELOT et al., 2007). Small sample volume ensures higher cooling rate and improves the vitrification efficiency (SARAGUSTY and ARAV, 2011). Unfortunately, vitrification affected piglet development in embryo transfer procedures. Vitrified embryo transfer produced only 12.3% piglets compared to 68.8% from fresh embryos (MISUMI et al., 2003). The low percentage of piglets derived from vitrified embryos may be due to selection of low quality oocytes and/or embryos for transfer.

Vitrification is a successful and useful cryopreservation technique for a long-term embryo preservation. Embryo faces damages after exposure to higher concentrations of cryoprotectants, and high cooling rate and warming procedures (HUANG et al., 2019). Also, more vitrified-warmed embryos are required for successful embryo transfer (ET) compared to fresh embryos (ŘIHA and VEJNAR, 2004). The blastocyst formation rates from vitrified morula is low in comparison to non-vitrified embryos (MANDAWALA et al., 2016). The vitrified pig embryos possess poor quality than fresh embryos, due to less number of cells (blastomeres) at blastocyst stage (KAMOSHITA et al., 2017) and high lipid content in the form of droplets i.e., 6.8 times in porcine than mouse (HUANG et al., 2019). These lipid droplets located in matured oocytes increase their chilling sensitivity (READER et al., 2017). The quality of vitrified-warmed

embryos influences the vitrification outcome (VAN LANDUYT et al., 2011). The selection of high-quality embryos without pathogens will improve biosecurity for pig producers. Transport of embryos, within and between countries, is safer and cheaper than live animals. In this experiment, the effects of vitrification of pig morulae, originated from sorted oocytes, on the subsequent quality of embryos, and development to blastocyst stage were studied. The objectives of this study were to evaluate: 1) the viability of vitrified pig embryos derived from sorted oocytes; and 2) the pregnancy rate following the embryo transfer of non-vitrified (fresh) and vitrified embryos in pre-pubertal gilts. Our hypothesis was *in vitro* produced pig embryos generated from sorted oocytes, based on mitochondrial and cortical granules distributions, survive better during vitrification process and yield pregnancy following transfer *in vivo*.

5.3. Materials and Methods

5.3.1. Animals and Chemicals

Ovaries of pre-pubertal gilts were collected from local slaughterhouses. Three live pre-pubertal piglets were used in embryo transfer experiment as a pilot experiment with animal care protocol number 20060052. All chemicals were purchased from Sigma-Aldrich Canada Co. (Oakville, Ontario, Canada) unless otherwise mentioned. All media were filtered with a 0.22 µm PVDF filter (Argos technology, Elgin, USA).

5.3.2. Oocytes Collection

Ovaries, collected from abattoir, were covered by thick cotton pad and placed into an insulated container (35-37 ºC) for transport to Westgen Lab, University of Saskatchewan. After removal of surrounding non-ovarian connective tissues, ovaries were washed (3X) in sterile saline (0.9% NaCl, at 22 °C) and immersed in glucose solution (0.128 M, at 22 °C) for 30 minutes. Cumulus oocyte complexes (COC) were collected from 3-8 mm follicles. Only grade 1 and grade 2 cumulus-oocyte complexes (COCs, with 3 or more cumulus cells layers and homogeneous cytoplasm) were selected for further processing. Oocytes were washed (3X) with washing medium (TCM-199, Invitrogen, Burlington, Canada; supplemented with 25 mM of HEPES and 50 mg/ml gentamycin; maintained at 37 ˚C).

5.3.3. *In Vitro* Oocyte Maturation

The COCs selected were rinsed once in maturation medium (TCM-199 supplemented with 3.05 mM D-glucose, 0.1% polyvinyl alcohol, 0.91 mM pyruvate, 0.57 mM L-cysteine, 10 ng/ml of Epidermal Growth Factor (EPG), 50 mg/ml of gentamycin and 5 µg/ml of luteinizing hormone (Lutropin-V®; Bioniche (Belleville, Canada)). The COCs were transferred in 4-well dish (500 µl of maturation of medium/well) and maximum 50 COCs were added per well, incubated for 44 hr in a controlled atmosphere (5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C in a humidified atmosphere).

5.3.4. *In Vitro* Fertilization

For *in vitro* fertilization, oocytes were divided into three groups: Group 1 (Standard IVF; control) – oocytes were matured following standard procedure using 10% porcine follicular fluid in maturation medium without sorting and staining and called non-sorted for further processing; Group 2 – oocytes were matured as control group and sorted based on mitochondrial (MT) distribution using 200 nM MitoTracker green (MTG) stain; and Group 3 – oocytes were matured as control group and sorted based on cortical granules (CG) distribution using 625 nM FITC-labelled peanut-agglutinin (PNA) stain. Oocytes possessing diffused mitochondrial pattern (Group 2) and peripheral cortical granules (Group 3) were selected for further processing and called as MT-sorted and CG-sorted oocytes hereafter (Figure 5.1). After completion of maturation, oocytes were denuded with a series of pipetting (approximately 80 times) using 0.1% hyaluronidase in TCM-199. Only mature oocytes possessing diffused pattern of mitochondria and peripheral pattern of cortical granules were selected for further *in vitro* fertilization. A modified Tris-buffer medium (mTBM) supplemented with 0.2% bovine serum albumin (BSA; Minitube, Verona, USA) and 1 mM of caffeine media was used for simultaneous sperm capacitation and *in vitro* fertilization. Frozen-thawed semen from an elite boar with proven fertility was used for fertilization. Straws were thawed at 37 °C for 20 seconds and diluted with mTBM media and centrifuged at 1000 × g for 5 minutes twice and resuspended in 1ml of mTBM. Motility and sperm concentration were evaluated using a Computer Sperm Assisted Analyzer (SpermVision, Minitube, Canada). Total of 50,000 motile sperm cells were incubated

with mature oocytes for six hours in a controlled environment (5% CO_2, 5% O_2 and 90% N_2) at 38.5 °C.

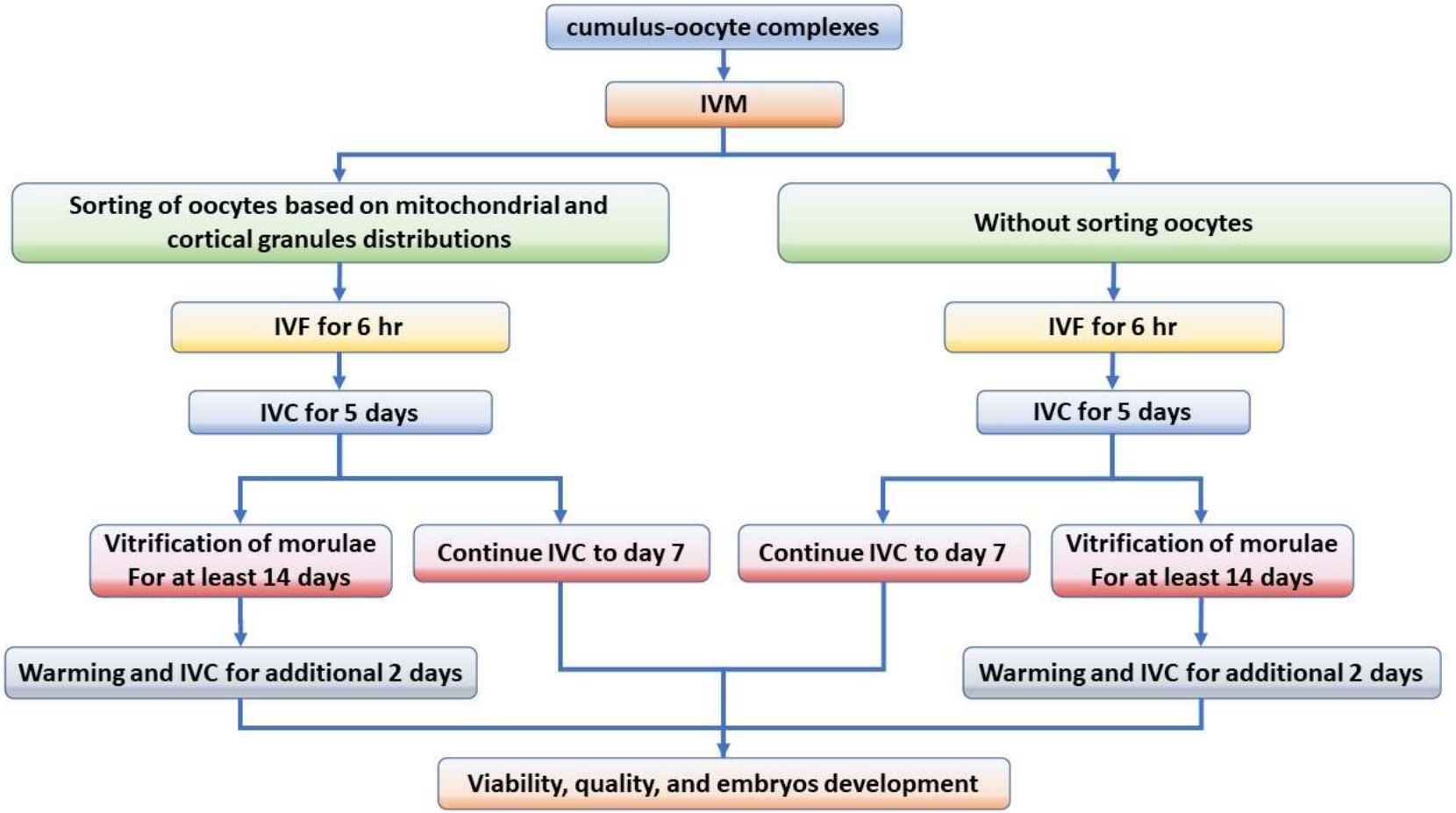

Figure 5.1: Experimental design for comparison of embryo quality from sorted and non-sorted oocytes without and with vitrification (Experiment 1). IVM: *in vitro* maturation, IVF: *in vitro* fertilization and IVC: *in vitro* culture.

5.3.5. Embryo Culture

Presumptive zygotes were washed (3X) with a modified NCSU 23 by replacing glucose with sodium lactate (4.5 mM) and sodium pyruvate (0.33 mM), then cultured in the same medium supplemented with 0.4% BSA in an environment of 5% CO_2, 5% O_2, and 90% N_2 at 37.5 °C, for 48 hr. Later, embryos were transferred to a regular NCSU 23 culture (with 5.55 mM of glucose) for additional 5 days. Cleavage rate and embryo development rates were assessed on day 3, 5, and 7 days. The quality of blastocysts produced was evaluated following the International Embry Transfer Society guidelines on day 7 of embryo culture. According to this evaluation, the quality grade 1 (Excellent or good quality) indicates to the individual blastomeres are homogeneous in size, color, and density to form a balanced and spherical mass in the embryos. This embryo is developing at the normal stage. Abnormalities should be minimal, and at least 85% of the molecules should be a surviving, complete embryonic mass. The quality grade 2 (Fair quality) indicates the general form of the embryonic mass, as well as the size, color, and density of single cells, are slightly irregular in these embryos. At minimum 50% of the embryonic weight must be undamaged. In quality grade 3 (Poor quality), these embryos demonstrate significant abnormalities in the form of the embryonic mass as well as in the size, color, and density of single cells. At least 25% of the embryo mass must be undamaged.

5.3.6. Experiment 1: Comparison of Embryo Quality in The Sorted and Non-Sorted Oocytes Before and After Vitrification

Bovine vitrification media (BOVIPRO vit kit from MOFA global; Ingersoll Ontario, Ontario, Canada) was used for vitrification of pig morulae. After maturation and *in vitro* fertilization of non-sorted control and MT- and CG-sorted oocytes, the morulae were transferred to the holding medium at 22 °C for 1 minute. The morulae were transferred into equilibration media A then B at 22 °C for 5 minutes each. After the exposure to equilibration media, morulae were transferred into the vitrification medium at 22 °C and then loaded in pre-labelled straws (5 embryos/straw), within 30 seconds. Each straw was exposed to liquid nitrogen vapor for 1 minute and then immersed in liquid nitrogen (Figure 5.2). In this experiment we selected the morulae based on quality. So, we omitted quality grade 2 and 3 for vitrification experiment. We selected only quality grade 1 for non-sorted, MT- and CG-sorted oocytes groups. After storage in

liquid nitrogen for at least 2 weeks, embryos were warmed for evaluation. Straws were kept in air at 22 °C for 10 seconds and transferred to a water bath (22-24 °C) for additional 10 seconds. Each straw was flicked (5-7 times) and kept in horizontal position for 6-8 minutes. The embryos were transferred from straw to the culture media for initial washing (3X; Figure 5.3).

All vitrified-warmed embryos were cultured for additional 2-3 days, and development was evaluated based on gross morphology of embryos under microscope. Embryo viability was tested with propidium iodide (PI; 1 mg/ml; ThermoFisher Scientific) in non-vitrified and vitrified embryos. Four groups were used in this experiment: Control 1 – fresh morulae from non-sorted oocytes; Control 2 – fresh morulae from MT- and CG-sorted oocytes; Treatment 1 – vitrified morulae from non-sorted oocytes; and Treatment 2 – vitrified morulae from sorted oocytes. In sorted groups, oocytes possessing diffused mitochondria and peripheral cortical granules were selected for further processing (Figure 5.3).

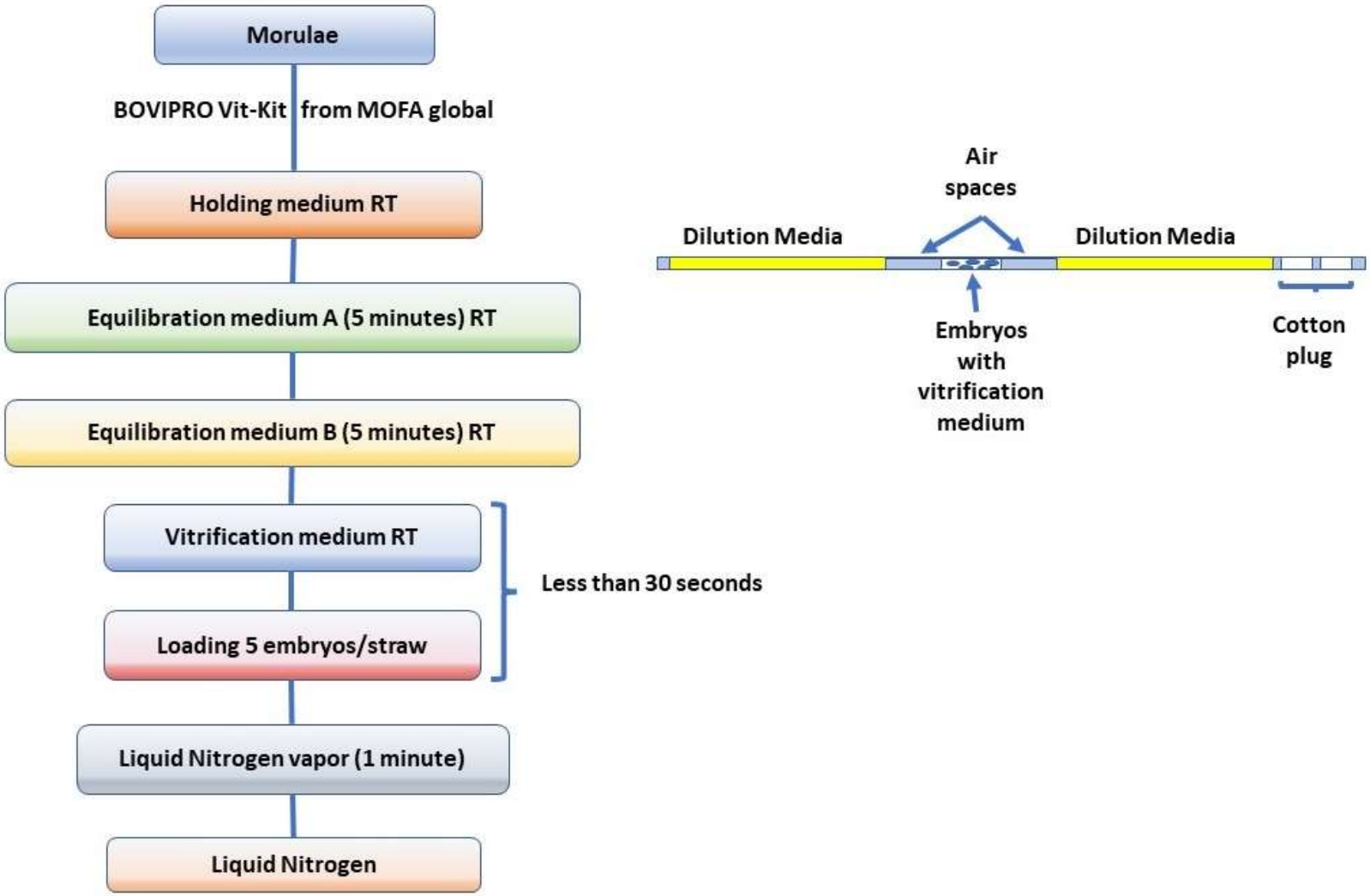

Figure 5.2: Schematic diagram representing the vitrification procedure for pig morulae (A) and the straw diagram showing embryos loading (B). RT: room temperature (22 °C)

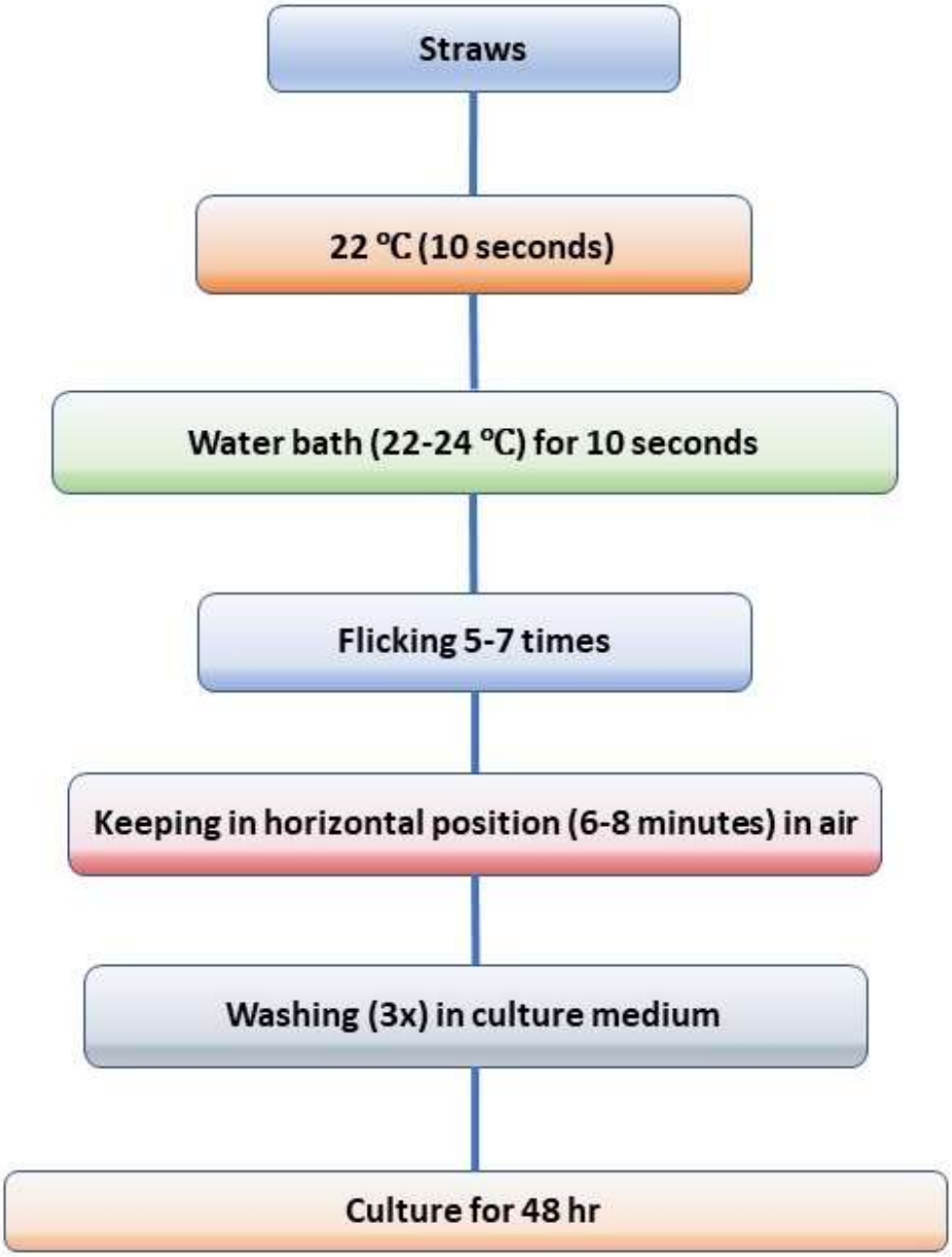

Figure 5.3: Schematic diagram representing warming procedure for pig morulae.

5.3.7. Experiment 2: *In Vivo* Transfer of Pig Embryo from the Sorted and Non-Sorted Oocytes

Three pre-pubertal gilts were used in this experiment. Each pre-pubertal gilt was given 7 ml canola oil orally, for 7 days by mouth, followed by 7 ml canola oil plus 7 ml of Regu-Mate solution 0.22% (Intervet Canada Corp., 16750 route Transcanadienne, Kirkland, QC, Canada), as synthetic progestin, for 18 days and 4 ml of Folligon (200 UI/ ml; Intervet Canada Corp., QC, Canada) as pregnant mare serum gonadotrophin (PMSG) 36 hr after last day of Regu-Mate administration. Embryos produced were divided into 2 groups: 1) Non-vitrified (control) – fresh *in vitro* produced embryos, and 2) Vitrified (Treatment) – embryo produced from non-sorted oocytes). Four days after Folligon treatment, the recipient pre-pubertal gilts expressed estrus. Five days after the onset of estrus that determined by estrus signs (swollen/red vulva), embryos were transferred into the tip of the uterine horn of pre-pubertal gilts. In both groups, embryos (n=30 each) were transferred by laparoscopic surgery into each pre-pubertal gilt by a board-certified veterinary surgeon. One gilt was used as a control (for non-vitrified embryos) and two gilts as treatment (for vitrified embryos). Low number of gilts was used due limited financial resources and this experiment was conducted as a pilot experiment. Pre-pubertal gilts were scanned with ultrasonography after 23, 63 and 78 days of surgical transfer. One pig of treatment group (vitrified embryos) was culled after the second scan because she returned to heat. After the end of experiment (98 days post transfer), the other two gilts were euthanized and a post mortem examination performed to confirm the pregnancy status and presence of corpora lutea on ovaries (Figure 5.4).

5.4 Statistical Analysis

Data from 3 or more replicates was compiled and analyzed using STATA 17.0 software (Copyright 1985-2021 StataCorp LLC, 4905 Lakeway Drive, College Station, Texas 77845 USA). In experiment 1, the percentage of oocytes or embryos within each repetition was the outcome variable used for all analyses. A zero-Skewness log transformation technique was applied before the statistical analysis to obtain a normal distribution. Differences across group were assessing using a one-way ANOVA was after the transformation. If significant, a pairwise comparison with Tukey's multiple group adjustment was used to confirm differences among groups. Data expressed the mean (%) ± standard deviation (SD). The minimum number of

embryos for this study is 60 based on the probability of 0.05 and experiment power of 90%. No statistical analysis done in experiment 2 due to low number of gilts used, as explained above.

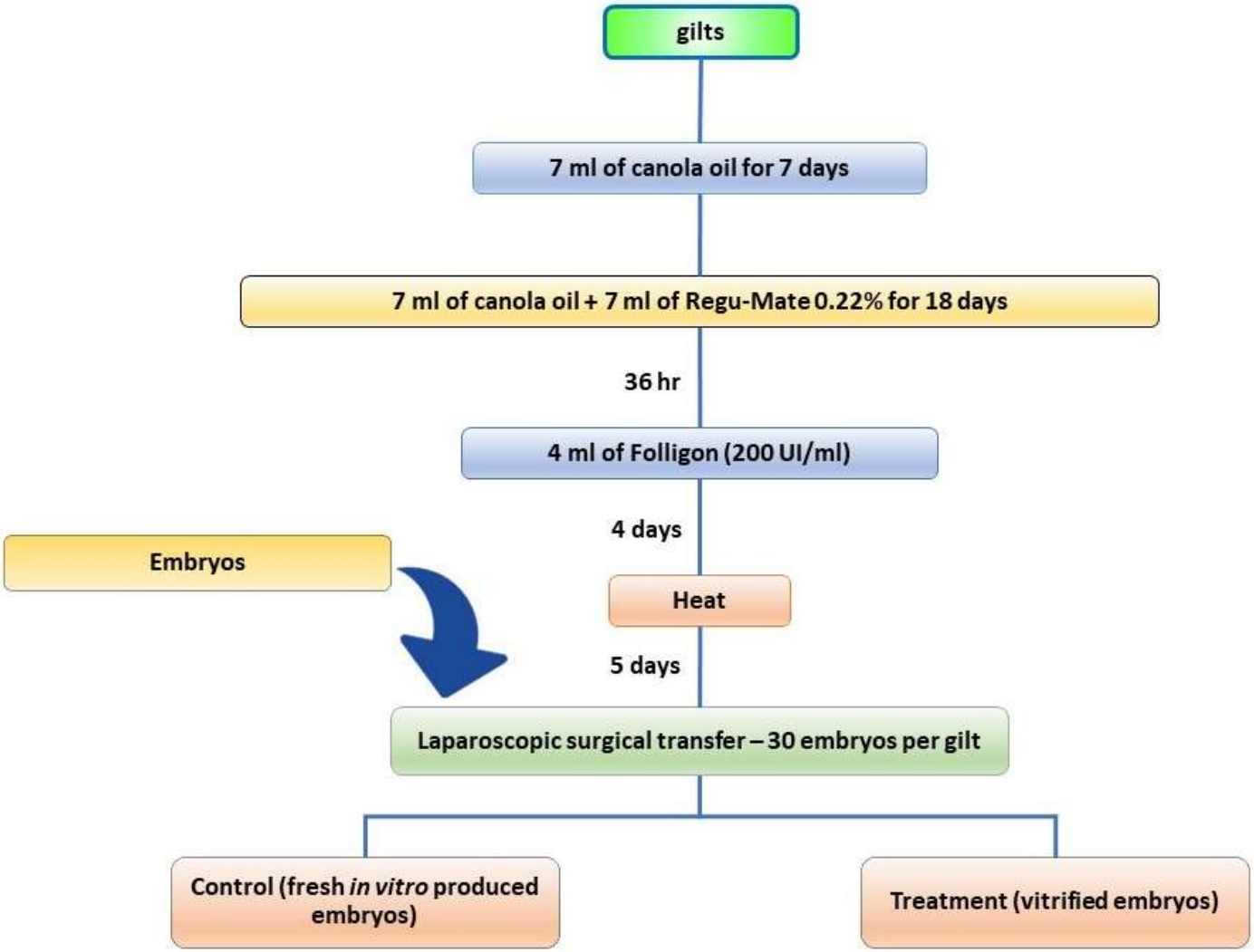

Figure 5.4: *In vivo* transfer of vitrified and non-vitrified pig embryos, originated from non-sorted oocytes, in pre-pubertal gilts (experiment 2).

5.5. Results

5.5.1. Experiment 1: Comparison of Embryo Quality in the Sorted and Non-Sorted Oocytes Before and After Vitrification

The viability rates of non-vitrified embryos, derived from MT-sorted oocytes (with diffused mitochondrial) and non-sorted oocytes, following IVF, were 98% ± 0.58 (44/45) and 100% ± 0.58 (44/44), respectively (Table 5.1). In contrast, the viability rates in vitrified-warmed embryos decreased significantly (P≤0.05) for both MT-sorted oocytes (42% ± 1.00, 27/65) and non-sorted oocytes (39% ± 1.53, 25/65) (Table 5.1). There was no significant difference in viability rates between the MT-sorted oocytes and non-sorted oocytes, within non-vitrified and vitrified embryos at morula stage.

Similar results were found in embryos originated from CG-sorted oocytes (with peripheral cortical granules) and non-sorted oocytes (Table 5.1). The non-vitrified embryos from CG-sorted oocytes and non-sorted oocytes, following IVF, had viability rates 100% ± 2.89 (40/40) and 98% ± 1.00 (42/43), respectively. However, the viability rate decreased significantly (P≤0.05) to 43% ± 1.15 (26/60) in vitrified embryos from CG-sorted oocytes and 40% ± 0.58 (22/55) in vitrified embryos from non-sorted oocytes. In contrast, within the non-vitrified and vitrified embryos, there was no significant difference in viability rates between sorted oocytes and non-sorted oocytes.

Table 5.1: The viability of non-vitrified and vitrified pig embryos derived from sorted oocytes (based on mitochondrial and cortical granules distributions) and non-sorted oocytes following standard IVF procedure. Each value represents mean ± SD.

Pattern	Viability of non-vitrified embryos		Viability of vitrified embryos	
	Total **	Rate (%) NS	Total	Rate (%) NS
Embryos derived from MT-sorted oocytes with diffused mitochondria *	45	98 ± 0.58 [a] (N=44) *** [100,100,93]	65	42 ± 1.00 [b] (N=27) [40,41,43]
Embryos derived from non-sorted oocytes *	44	100 ± 0.58 [a] (N=44) [100,100,100]	65	39 ± 1.53 [b] (N=25) [35,42,38]
Embryos derived from CG-sorted oocytes with peripheral cortical granules *	40	100 ± 2.89 [a] (N=40) [100,100,100]	60	43 ± 1.15 [b] (N=26) [40,40,50]
Embryos derived from non-sorted oocytes *	43	98 ± 1.00 [a] (N=42) [100,93,100]	55	40 ± 0.58 [b] (N=22) [35,47,40]

*Within a pattern of oocytes (row), values with different letters (a and b) represent differences ($P \leq 0.05$) in embryos viability.
**Total number of embryos used
***N represents number of embryos found viable.
NS represents non-significant difference in a column.
The numbers between square brackets represent the percentage of each replicate.

Total 373 morulae were produced *in vitro* and subjected to quality evaluation without and after vitrification (Table 5.2). In MT-sorted and non-vitrified group, 83% ± 1.00 (33/40) morulae had grade 1 and 81% ± 1.53 (34/42) from non-sorted and non-vitrified group; 12% ± 0.58 (5/40) and 12% ± 1.15 (5/42) grade 2 quality embryos, and 5% ± 0.58 (2/40), 7% ± 0.58 (3/42) grade 3 morulae, respectively. In vitrified group, the number of grade 1 morulae decreased ($P \leq 0.05$) to 2% ± 0.58 (1/50) for both MT-sorted and non-sorted groups. Correspondingly, the grade 3 morulae increased ($P \leq 0.05$) to 82% ± 1.53 (41/50) and 84% ± 1.73 (42/50), respectively. However, there was no significant change in grade 2 quality morulae (16% ± 1.53, 8/50 and 14% ± 0.58, 7/50, respectively). In all grade 1, 2, and 3 morulae, there was no difference between the sorted and non-sorted groups, within non-vitrified and vitrified groups.

Similar results were found in morulae derived form CG-sorted oocytes (Table 5.2). In non-vitrified group, 87% ± 1.00 (39/45) morulae from sorted oocytes and 85% ± 1.00 (39/46) of morulae from non-sorted oocytes had grade 1 quality, 9% ± 0.58 (4/45) and 11% ± 0.85 (5/46) of grade 2 quality respectively, and 4% ± 0.58 for both groups (2/45 and 2/46) of grade 3 quality. In vitrified group, the number of grade 1 morulae decreased significantly ($P \leq 0.05$) to 4% ± 1.15 (2/50) and 2% ± 0.58 (1/50) for the CG-sorted and non-sorted groups, respectively. Correspondingly, the grade 3 morulae increased significantly ($P \leq 0.05$) to 78% ± 1.00 (39/50) and 82% ± 2.08 (41/50) for CG-sorted group and non-sorted group, respectively. However, there was no significant change in grade 2 morulae 18% ± 1.00 (9/50) and 16% CG-sorted and non-sorted groups, respectively. In all grade 1, 2, and 3 morula, there was no significance difference between the CG-sorted and non-sorted groups, within non-vitrified and vitrified groups.

Table 5.2: The quality of non-vitrified and vitrified pig embryos derived from sorted oocytes (based on mitochondrial and cortical granules distributions) and non-sorted oocytes following standard IVF procedure. Each value represents mean ± SD.

Pattern	Quality of non-vitrified embryos				Quality of vitrified embryos			
	Total **	Grade 1 (%) NS	Grade 2 (%) NS	Grade 3 (%) NS	Total	Grade 1 (%) NS	Grade 2 (%) NS	Grade 3 (%) NS
Embryos derived from MT-sorted oocytes with diffused mitochondria *	40	83 ± 1.00 [a] (N=33) *** [83,85,80]	12 ± 0.58 [b] (N=5) [8,15,13]	5 ± 0.58 [bc] (N=2) [8,0,7]	50	2 ± 0.58 [c] (N=1) [0,5,0]	16 ± 1.53 [b] (N=8) [7,20,20]	82 ± 1.53 [a] (N=41) [93,75,80]
Embryos derived from non-sorted oocytes *	42	81 ± 1.53 [a] (N=34) [77,85,81]	12 ± 1.15 [b] (N=5) [8,8,19]	7 ± 0.58 [bc] (N=3) [8,8,0]	50	2 ± 0.58 [c] (N=1) [5,0,0]	14 ± 0.58 [b] (N=7) [15,13,13]	84 ± 1.73 [a] (N=42) [80,87,87]
Embryos derived from CG-sorted oocytes with peripheral cortical granules *	45	87 ± 1.00 [a] (N=39) [93,80,87]	9 ± 0.58 [b] (N=4) [7,13,7]	4 ± 0.58 [bc] (N=2) [0,7,7]	50	4 ± 1.15 [c] (N=2) [10,0,0]	18 ± 1.00 [b] (N=9) [20,20,13]	78 ± 1.00 [a] (N=39) [70,80,87]
Embryos derived from non-sorted oocytes *	46	85 ± 1.00 [a] (N=39) [80,88,87]	11 ± 0.85 [b] (N=5) [13,13,7]	4 ± 0.58 [bc] (N=2) [7,0,7]	50	2 ± 0.58 [c] (N=1) [0,0,5]	16 ± 0.58 [b] (N=8) [13,20,15]	82 ± 2.08 [a] (N=41) [87,80,80]

*Within a pattern of oocytes (row), values with different letters (a-c) represent differences (P≤0.05) in morulae rate.

NS represents non-significant difference within a column.

**Total number of embryos used

***N represents number of embryos found in different grades.

The numbers between square brackets represent the percentage of each replicate.

In the non-vitrified (fresh) group, 15% ± 1.00 (6/40) and 12% ± 0.58 (5/42) morulae developed to blastocysts stage in MT-sorted and non-sorted groups, respectively (Table 5.3). No blastocyst developed from vitrified morulae in both MT-sorted and non-sorted groups. This reduction was significantly less ($P \leq 0.05$) than the non-vitrified group. In addition, there was no significant difference between the MT-sorted group and non-sorted group, within non-vitrified and vitrified groups.

According to cortical granules sorting, similar results were found (Table 5.3). In non-vitrified group, 16% ± 0.58 (7/45) of morulae from CG-sorted group and 13% ± 0.00 (6/46) from non-sorted group developed to blastocyst stage. The blastocyst development dropped to 0% ± 0.00 (0/50) after the vitrification in both non-vitrified and vitrified groups. There was no significant difference between CG-sorted and non-sorted, within non-vitrified and vitrified groups.

Table 5.3: The development of embryos (morulae to blastocysts) derived from sorted oocytes (based on mitochondrial and cortical granules distributions) and non-sorted oocytes following standard IVF procedure. Each value represents mean ± SD.

Pattern	Embryos development without vitrification		Embryos development after vitrification and warming	
	Total morulae **	Blastocyst formation rate (%) NS	Total morulae	Blastocyst formation rate (%) NS
Embryos derived from MT-sorted oocytes with diffused mitochondria *	40	15 ± 1.00 [a] (N=6) *** [8,15,20]	50	0 ± 0.00 [b] (N=0) [0,0,0]
Embryos derived from non-sorted oocytes *	42	12 ± 0.58 [a] (N=5) [8,15,13]	50	0 ± 0.00 [b] (N=0) [0,0,0]
Embryos derived from CG-sorted oocytes with peripheral cortical granules *	45	16 ± 0.58 [a] (N=7) [13,13,20]	50	0 ± 0.00 [b] (N=0) [0,0,0]
Embryos derived from non-sorted oocytes *	46	13 ± 0.00 [a] (N=6) [13,13,13]	50	0 ± 0.00 [b] (N=0) [0,0,0]

*Within a pattern of oocytes (row), values with different letters (a and b) represent differences ($P \leq 0.05$) in blastocysts.
**Total number of morulae used
***N represents number of embryos found at blastocyst stage.
NS represents non-significant difference in a column.
N represents number of embryos used.
The numbers between square brackets represent the percentage of each replicate.

5.5.2. Experiment 2: *In Vivo* Transfer of Pig Embryos from the Sorted and Non-Sorted Oocytes

Out of three, one pre-pubertal gilt from treatment (vitrified embryo) group was found non-pregnant as she returned to heat after the second scan of ultrasonography (day 63 post-transfer) and thus was culled. There was some uncertainty regarding pregnancy status of the other two gilts because they expressed pregnancy signs like enlargement of mammary glands and abdomen, and did not return to heat, so they were scanned for the third time. Unfortunately, no pregnancy was detected in these pigs after the third scan (Table 5.4). After the necropsy, the first pig from the treatment group was found to be not pregnant and there was no corpus luteum on ovaries. On the other hand, the other pre-pubertal gilt had several corpora lutea. The control pig did not have any corpus luteum on ovaries (Table 5.5).

Table 5.4: Pregnancy outcome in pre-pubertal gilts after transfer of control and vitrified embryos.

Gilt #	Treatment	First scan	Second scan	Third scan
1	Control (fresh embryos)	NP *	NP	NP
2	Treatment (vitrified embryos)	NP	NP	Culled **
3	Treatment (vitrified embryos)	NP	NP	NP

*NP indicates non-pregnant.
**One of the treatment pigs was culled because she came into heat.

Table 5.5: Necropsy of pig for detection of pregnancy and corpora lutea.

Gilt #	Treatment	Pregnancy	Corpora lutea
1	Control (fresh embryos)	NP *	NP
2	Treatment (vitrified embryos)	NP	NP
3	Treatment (vitrified embryos)	NP	CL **

*NP means neither pregnancy nor corpus luteum.
**CL means corpora lutea.

5.6. Discussion

The conservation of endangered breeds can be achieved by storing the number of pure breed embryos using the assisted-reproductive techniques like *in vitro* fertilization (IVF), vitrification and transfer of embryos. These techniques can help to select embryos with high quality and biosecurity. This study was conducted to investigate the effect of sorting of oocytes based on cytoplasmic maturation indicators (diffused mitochondria and peripheral cortical granules) on the embryos outcomes (viability, quality and development *in vitro*). Also, we transferred the non-vitrified and vitrified embryos to compare further development *in vivo*.

The viability of vitrified-warmed embryos, originated from MT-sorted oocytes was 42% in comparison to 39% of non-sorted oocytes, with no significant difference. In addition, the viability rate of vitrified-warmed embryos, originated from CG-sorted oocytes, was 43% in comparison with 40% vitrified embryos derived from non-sorted oocytes. These low viability rates were dramatically less than fresh embryos (98-100%). The viability rate of embryos may depend on the stage of embryos at which they are vitrified and the vitrification procedure used. Usually the morula stage has lower viability rates (56-67.6%) than the blastocyst stage (74-95.5%) because of the high lipid content in morulae compared to blastocysts (CUELLO et al., 2004, USHIJIMA et al., 2004, BRUSENTSEV et al., 2019). The higher lipid content increase sensitivities of embryos to cooling (ZHOU and LI, 2009). The high content of lipid droplets in early stages of embryo development such as morula can cause an osmotic stress due to high concentration of cryoprotectant (MEN et al., 2005). To reduce the lipid content of embryos, delipidation (lipid droplets removal) before the vitrification-warming procedure is the solution to increase the embryonic viability and development (MEN et al., 2015, XINGZHU et al., 2021). The lipid content in pigs is about 6.8 times higher than in mouse and about 4 times higher than in cattle oocytes (GENICOT et al., 2005). This high content of lipid causes the cryoinjury in embryos. Lipid droplets will cluster and enlarge when pig blastocysts are chilled to +15 ˚C, resulting in embryonic mortality (AMSTISLAVSKY et al., 2019). This temperature is the lipid phase transition in pig embryos (PRATES et al., 2014). Researchers think that the lipid phase transition is the primary cause of cell damage at temperatures below the optimal but above the freezing point, and phospholipids in membranes are redistributed by lipid phase transition and this might lead to their damage (AMSTISLAVSKY et al., 2019).

The system used in this study was closed system using in 0.25 mm straws. This system is less successful in survival rate (20%) than other systems like gel loading tip (39%) (USHIJIMA et al., 2004) or open pulled straws (56.3%) (CUELLO et al., 2004). The cryopreservation in closed system can be successful only with removal of lipid content from oocytes (lipidation) (ZHANG et al., 2012). However, the open system is more likely to expose the contamination from the liquid nitrogen (AMSTISLAVSKY et al., 2019). On the other hand, previous results from our lab had similar results between closed and open systems (AL-SHANOON, 2016). The concentration of cryoprotectants used in vitrification system is another important factor due to associated toxicity is an important factor in success or failure of vitrification process (BOJIC et al., 2021). The high concentration of cryoprotectants leads to induce toxic effect on the vitrified embryos (USHIJIMA et al., 2004). In this study, the vitrification and warming of pig embryos was conducted with a commercial kit using a standard cryoprotectant concentrations for bovine embryos.

The number of embryos with grade 1 (the best quality) decreased from 81-87% in non-vitrified (fresh) embryos to 2-4% in vitrified-warmed embryos. This decrease may be caused by the sensitivity of pig embryos to the temperature change and sensitivity to chilling temperature due the high lipid content, as described above.

Also, the failure in developing the embryos to blastocyst (0%) could be the result of sub-optimal culture conditions for the *in vitro* embryo production system. *In vitro* production of embryos in pigs and the quality of embryos are still affected by culture system used and media (GRUPEN, 2014) which is lower than that observed in the bovine species. The mean IVEP blastocyst formation in pigs is approximately 30% under the best conditions (FOWLER et al., 2018).

Another possible factor for poor embryonic development might be the oocyte source used in this study. Embryos were obtained from pre-pubertal gilts in this study. Oocytes from pre-pubertal gilts showed lower competence compared to adult sows (GRUPEN et al., 2003) resulting in the low development of the embryo (GRABOWSKA et al., 2016). Poor embryo development might be due to insufficient cytoplasmic maturation of oocytes or high polyspermic (GILCHRIST and THOMPSON, 2007).

Successful embryos transfer is the main target of porcine industry. There are many factors which can affect the outcome of embryo transfer. The main reason of failure of embryos transfer of this study is the small number of gilts used in this experiment. One pig was used for fresh embryos and two pigs for vitrified embryo transfers because of limited financial resources for a larger trial. Another reason of the failure of embryo transfer for the vitrified-warmed group was poor viability rate. Surprisingly, there was a lack of pregnancy in fresh embryo transfer group also. In this study, this was the first experience of the surgeon for surgical embryo transfer in pigs and he used an unproven laparoscopic technique. Also, the blastocyst development in low rate in our lab was another possible reason of poor conception rate *in vivo*. Morulae has less viability in embryo transfer than using the blastocysts (ANGEL et al., 2014). Finally, the site of embryo deposit within the uterus should be suitable for embryos viability, so it is important to know the exact site according to the pig embryonic stage (MARTINEZ et al., 2019).

One of the gilts had many corpora lutea in the ovary. There is evidence that the corpora lutea can release hormonal factors that effect on endometrial secretions in the lumen of uterus to maintain pregnancy (ANGEL et al., 2014).

5.7. Conclusions

The vitrification-warming procedure negatively affected the survival rate, quality, and blastocyst formation of embryos. The low viability rate may be caused by the embryos being overly sensitive to cooling due to high content of lipid in pig oocytes and embryos or the closed system applied with 0.25 mm straws. The low rate of blastocyst formation and the surgeon's first experience performing laparoscopic ET in pigs may be the main reasons for the embryo transfer failure.

Some modifications in cryoprotectants and their concentration, packaging system, cooling velocity will be needed to develop a comprehensive vitrification-warming procedure, for successful cryopreservation and transfer of pig embryos, for conservation purposes. More experiments will be needed to evaluate different commercial media used in vitrification-warming. A larger number of animals are required for embryo transfer. Other modifications such as using blastocysts instead of morulae or using dilapidation procedure before the vitrification to reduce the lipid droplets content in embryos would be worth trying.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

6.1. General Discussion

In this dissertation, we evaluated the oocyte viability with different fluorescent dyes specific for assessment of mitochondria and cortical granule distribution (MTO, MTG, RH-123, PNA, and WGA) on oocytes. Also, we examined the distributions of MT and CGs using MTG and PNA respectively, under confocal and wide-field microscopy. Results from this dissertation confirmed that MTG and PNA dyes were safe to pig oocytes (chapter 2). Our sorting techniques based on cytoplasmic maturation of porcine oocytes was found useful to produce high quality pig embryos with less polyspermy (chapter 3). We found that the maturation media used in this dissertation did not affect pig embryo development (chapter 4). In addition, the use of sorted (mature) oocytes did not improve the embryo survival, quality, and development after vitrification-warming procedures. Rather, vitrification and warming procedures per se negatively impacted embryonic development. Also, the transfer of fresh and vitrified embryos did not produce any pregnancy (chapter 5).

Initial experiments were conducted to examine the oocyte viability using different fluorescent dyes specific to mitochondria and cortical granules (MTO, MTG, RH-123, PNA, and WGA), varying in concentrations, on the live oocytes and the distribution patterns of MT and CGs. The distribution of MT and CGs, using MTG and PNA respectively, was examined under the wide-field and confocal microscopy. Viability results of oocytes showed that there was no significant harm effect of fluorescent dyes on pig oocytes, and MTG with a concentration of 200 nM and PNA with a concentration of 625 nM were selected for subsequent experiments. Moreover, clear and visible distributions of MT and CGs could be observed using the wide-field microscope.

In the second series of experiments, the pig oocytes (from adult sow and pre-pubertal gilts) were sorted based on MT and CG distributions. Oocytes with diffused MT and peripheral CG were sorted and used for further investigations. *In vitro* maturation, embryonic development

and polyspermy of live, and sorted and non-sorted oocytes derived from adult sows and/or pre-pubertal gilts was determined. Blastocyst rates were statistically equivalent between sorted and non-sorted oocytes. Embryo production is significantly affected by polyspermy in porcine embryos (GRUPEN, 2014). The exocytosis is a landmark of oocyte maturation. Immature oocytes were more likely to have polyspermy, whereas mature oocytes with peripheral CGs controlled the number of sperm entering oocytes. Adult sow oocytes were fully capable of translocating CGs before and after maturation, which is only partially present in prepubertal oocytes. The actin filament helps the translocation of CGs (LIU, 2011, CHEESEMAN et al., 2016a). Whereas mitochondria are transported through microtubules (YAMOCHI et al., 2015).

Follow up experiments were performed to evaluate the effect of different maturation media (TCM-199, mWM, NCSU23, NCSU37G and NCSU37PL) on MT and CGs distribution, embryos development, and polyspermy. There was a higher rate of diffused MT pattern in TCM, mWM, and NCSU37PL media than other two media. All these three media have pyruvate in their chemical composition. Pyruvate is required for mitochondrial metabolism during development, and it helps the MT to use adenosine triphosphate (ATP) more quickly (UDAGAWA and ISHIHARA, 2020). TCM media showed a higher peripheral pattern of CGs. TCM includes more calcium (1.7 mM/L vs. 1.8 mM/L; Table 4.1), acting as a stimulating component in CGs translocation (FERREIRA et al., 2009). Morula stage embryos were higher in TCM for both non-sorted and CG-sorted oocytes than the other media. Glucose uptake increases during embryo development, particularly in the morula or blastocyst stage (JONES et al., 2001). Polyspermic fertilization was lower in TCM because of the high concentration of pyruvate. Polyspermy rates were affected by the presence or absence of peripheral CGs. Pyruvate inhibits early exocytosis of cortical granules and decreases the time to dissolve zona pellucida and thus enhanced fertilization by delaying oocyte ageing (LIU et al., 2009). The sorted (matured) oocytes and non-sorted oocytes had a better outcome in successful fertilization than the immature oocytes in all the media utilized.

In the last series of experiments, we evaluated the effect of sorting oocytes on the viability, quality, and development of embryos after the vitrification and warming procedures. Also, we transferred vitrified or non-vitrified (fresh) embryos in live pre-pubertal gilts. Embryo viability rates were not significantly different between MT-sorted, CG-sorted and non-sorted oocytes. The main factor responsible for the failure of vitrification is the high lipid content in pig

embryos (GAJDA, 2009). High lipid content makes the embryos more sensitive to chilling (NOHALEZ et al., 2018b). Embryo transfer of both non-vitrified and vitrified embryos did not yield pregnancy. The laparoscopic surgical embryo transfer procedure was performed by the surgeon for the first time on pigs. *In vitro* blastocyst rate was also poor, which was reflected *in vivo*. Embryo stage and the site of transfer in female tract is also important. Using morulae instead of blastocysts results in a lower rate of survival (ANGEL et al., 2014).

The safeness of dyes through maturation encouraged us to use this technique for embryo sorting in the subsequent experiments. The sorting of oocytes under a simple wide field microscope, for cytoplasmic maturation, was more for the evaluation of pig oocytes, as compared to the confocal microscope. Using MTG for mitochondria and PNA for CG on live oocytes instead of fixed oocytes was the greatest strength of this study. This is the first report of its kind using fluorescent dyes to determine the cytoplasmic maturation of live pig oocytes. All previous research was done on fixed oocytes (PAWLAK et al., 2016a, SONGSASEN et al., 2017, CUI et al., 2019, KULUS et al., 2020). The quality of the blastocysts produced was better in the sorted group than the non-sorted oocytes. In any case, increasing the quality of *in vitro* developed blastocysts would be useful for projects related to production of healthy porcine embryos, for conservation purposes. Oocytes possessing similar cytoplasmic maturation will have same autocrine/paracrine factors for maturation, fertilization and embryo development (WYDOOGHE et al., 2017, MACHTINGER et al., 2016).

In this study, a single MTG or PNA marker was used at a time to determine the cytoplasmic maturation, so the sorting oocytes did not enhance the production of embryos; therefore, it is recommended to use simultaneous MT- and CG based sorting procedure for future studies. The blastocyst rate in this study was low compared to earlier studies (ISOM et al., 2012, GIL et al., 2013, MARTINEZ et al., 2015a). Vitrified embryos survival, quality, and development were significantly lower than non-vitrified embryos. The viability of vitrified embryos is affected by the embryonic stage at the time of vitrification. The morula stage has a lower survival chance than the blastocyst stage (CUELLO et al., 2004, USHIJIMA et al., 2004). Morulae and early cleaved embryos have more lipid content than pre-hatched blastocysts (BRUSENTSEV et al., 2019), hence, morulae are more sensitive to cooling injury during vitrification (ZHOU and LI, 2009). Osmotic stress associated with high concentrations of cryoprotectants, early stage of embryo, such as morula, because of the high concentration of lipid

(MEN et al., 2005). In future experiments, it is recommended to use large number (minimum of 10 animals/group) of pre-pubertal gilts or adult sows for *in vivo* fertility trial.

This research has added new information to the pool of knowledge on using cytoplasmic maturation markers with least harmful effect for the production of high-quality embryos, transferable *in vivo*. One of the problems in porcine industry is low *in vitro* production of embryos (IVEP) outcome, due to the lack of information about cytoplasmic maturation (TREBICHALSKA et al., 2021) and relevant markers for detection.

Our recommendation is to stain pig oocytes with 200 nM of MTG and 625 nM of PNA and utilise a wide-field (inverted) fluorescence microscope instead of the normal wide-field fluorescence microscope for a simple and rapid way to separate pig oocytes by MT and CGs distribution. In the future studies, mixed fluorescence dyes could be used to enhance the IVEP procedure such as MTG with PNA or MitoTracker red with PNA to increase the chance of simultaneous MT- and CG- based sorting. Developing vitrification-warming media could improve the vitrification outcome if a porcine specific media is used. Our final recommendation is to use blastocysts instead of morulae for embryos transfer in a larger number of pigs to evaluate the success of IVEP.

6.2. General Conclusions

1. There was a little or no harmful effect of dyes tested on the viability of live pig oocytes.
2. There was a strong agreement between a wide-field microscope and confocal microscope in terms of assessment and sorting of oocytes (>96%), based on cytoplasmic maturation. Therefore, a wide-field microscope can be used because it is simple to operate and yield quick results.
3. The MT- or CG-based sorted oocytes yield better-quality embryos *in vitro*.
4. *In vitro* fertilization study, the sorted oocytes had a lower polyspermy rate.
5. Media with pyruvate supplement produced more matured oocytes.
6. Maturation media had no significant effect on the production of blastocyst stage embryos.
7. A comprehensive protocol is required for efficient vitrification and warming of pig oocytes (e.g. vitrification and warming media, concentrations of cryoprotectants, packaging system, number of embryos in a batch, volume of sample etc.).
8. A larger number of pigs will be required for successful embryo transfer.
9. The laparoscopic surgery protocol needs to be fully developed and tested on fresh embryos before transfer.
10. A limitation of the research is the low blastocyst rates. Improving blastocyst rates in the future may be important to the success of ET.

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APPENDIX A

IMARIS SOFTWARE TO MEASURE MITOCHONDRIAL DISTRIBUTION PATTERNS AND CORTICAL GRANULES DISTRIBUTION OF PORCINE OOCYTES.

Methods:

This method is specialized for mitochondrial (MT) distribution. About cortical granules (CGs) distribution, just change the names of MT and the value in step d) 6 to 0.2 since the diameter of cortical granules is 0.2 µm. Also change the threshold in step f) the second method #2 from 10 to 2 as the distance to the surface is 2µm.

Note: All words within quote marks are exactly in Imaris software as Imaris terminology.

- Using AutoQuant X3 to do deconvolution of confocal images.
- In Imaris 8.1.2 software: To identify MT distribution (clusters or individuals):

a) Threshold Cutoff:

1. Image processing
2. Thresholding
3. Threshold cutoff
4. Choose the channel (channel 1)
5. The values below should be 0.
6. To keep the noise level to the minimum, we should see all images before starting. In my case most of images had MT signals between the slices 10 and 60 of the oocyte (started at 10 and decreased at 60).

b) Surface Build Up:

1. New surface 
2. Uncheck “segment only a Region of Interest”.
3. Click on “Skip automatic creation, edit manually”.

4. Go to “Draw tab” 
5. Under contour tab: go to “board” tab, and under orientation select XY, and under “Resolution” check the “Manual” and chose 512x512, and then check “preserve features”. Within “Board visibility”, choose “Next” for more accurate.
6. Under contour also, but within “Mode”, click on “Distance drawing”  , and change the “Vortex spacing” to 0.5 um.
7. Within “Mode” also, click on “Circle” button  , and within parameters, change the “Number of vertices” to 100. This number will give you a good circle shape.
8. Rotate the image from back to front to see the surface of oocyte. Do that every time you want to recognize the surface.
9. From the right side of screen within Camera/labels- Camera- Pointer choose “Select” instead of Navigate and from “Draw tab” click on “Draw” button in the left down of the screen

10. Starting with signal appearing (in my case after the slice number 10) draw contour lines surrounding the signals under the plasma membrane. Start from the slice number with the first signal and end with slice number of last signal with intervals of 2 slices for each contour line. Rotate the 3D view if necessary.
11. Click on “Create surface” button.
12. Under “edit tab” (the pencil icon ) click on “Mask All…” button.
13. In Mask channel pop up screen: choose the channel and check the “Duplicate channel before applying mask”. Within “Mask Settings”, select “Constant inside/outside:”, and check the “Set voxels outside surface to:” and make the value as 0. Click OK button. The voxel means the volume and element of the object.

c) Transformation of Distance to Surface:

1. To calculate the distance properly, the data must be 32-bit float image. If it is not, go to Edit, and then select “Change Data Type”. In this pop-up screen, change to 32-bit float image.
2. Select the surface.
3. Click on the “Tools” button  .

4. Select “Distance transformation”.
5. In pop up screen, select “Inside SurfaceObject” and then click “OK”. Wait until finished. This will create “Distance to surfaces” channel. This channel’s intensity means the shortest distance from a Surface/Spots object border. On the other hand, this channel contains distance information. We selected “Inside SurfaceObject” option because we need to measure the contents inside the surface that we build (MT).

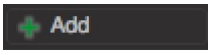
d) Identification of MT as Spots:

1. Click on “Add new spots” button  .
2. Rename by double click on it as “MitoAll”
3. Under the spots, within create tab 

, Uncheck “Segment only a Region of Interest”, and check “Different Spot Sizes (Region Growing)”. We unchecked the first option because we need the computation to be repeated for the entire image (different regions). The second option lets the region grows until a border stops the growing.
4. Click “next”  .
5. Select the Masked channel because we need to compute the spots inside the surface (oocyte) not outside.
6. Put the value of 1 um within “Estimated XY Diameter” since the MT diameter is 1 µm.
7. Check the “Background Subtraction” to remove the background prior to spot detection.
8. Click next  .
9. Within quality filter change the slider position to select all spots inside the cytoplasmic membrane.
10. Click next  .
11. Under “Spot Regions form”, select “Local Contrast” because the Absolute Intensity method does not correctly segment all spots.
12. Click next  .
13. Under “Spot Regions”, select “Region Volume”. In this option, the spots radius is measured from the region volume (the spots volume and the region volume are equal).

14. Click on “finish” button  .
15. Under “Points Style / Quality”, select “Sphere” to view the spots as bubbles.

e) Individual MT:

1. Click on “spots tools” button  .
2. Select “Spots to Spots Closest Distance” to calculate the distance between two closest spots.
3. Within pop up screen, select “Spots Statistics” (to compute each spot depending on a spatial position throughout the x, y, and z axis as statistics such as distance max, distance mean, distance min) and “Spots Radius” (to determine the maximum intensity to the voxel at the center of the spots).
4. Click “OK”.
5. Please, wait until the processing complete, and then click on “Center” to calculate previous measurement from the center of the spots not from the border.
6. This will create new spots (“MitoAll Dist”).
7. Choose “MitoAll Dist” spots.
8. Choose the filter tab  .
9. Click on “Add” button  .
10. Within “Filter Type” select “DistMin”
11. Click on the “Upper threshold” button and put the value of 2 um to select the bubbles that are less than 2 micron  , and then click on the “Lower Threshold” button to turn it off (first button). This step to determine the distance between the clusters no more than 2 microns. The selected bubbles (yellow) are clusters, while the green bubbles are the distance between the clusters in next step.
12. Click on “duplicate Selection to new spots” to create clustered bubbles as “MitoAll Dist Selection”  .
13. Change the name of “MitoAllDist Selection” to “Clusters”. These spots are the total number of clusters.

f) Identification of Central or Peripheral MT.

There are two methods to determine the position of MT. After doing a comparison between the first method and the second method, the 1st method is more accurate than the 2nd method to represent the clusters. So, dismiss it in CGs measurements because there are no clusters.

- The first method:

1. Select “MitoAll” spots
2. Go to the tools .
3. Select “Find Spots Close to Surface”
4. Choose “MitoAll” and “Clusters”
5. Click “OK”.
6. In the pop-up screen “Please enter the threshold” put 10 as a distance of 10 µm to the surface.

- The second method:

1. Select the “MitoAll” spots and add filter (Intensity Center Ch=3). This channel is “Distance to Surface1”
2. Turn off the “lower threshold” and click on “upper threshold” to put the value of 10. This value is the distance of 10 um to the surface 

.
3. Click on “duplicate selection to new spots” 

. This will create new spots (“MitoAll Selection”). These are peripheral MT.
4. Do the same steps from 1 to 3 by choosing “MitoAllDist Selection” to get peripheral clusters.