Influences of Delayed Meiotic Resumption on Porcine Oocyte Competency

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ABSTRACT

In pigs, successful *in vitro* production of embryos (IVP) faces significant challenges due to many defects in various steps of this technique, including: *in vitro* maturation of oocytes, *in vitro* fertilization, and *in vitro* culture of embryos. Poor cytoplasmic maturation of oocytes is considered the main factor for low rate of IVP of pig embryos. One of the main causes of this problem is the insufficient time for cytoplasm of oocytes to be matured due to the loss of communications between cumulus cells and oocytes. Recently, a new strategy emerged to inhibit meiotic resumption and provide more time to accumulate important factors for oocytes competence. The overall hypothesis of this thesis was that delaying meiotic resumption during the *in vitro* maturation will have a positive effect on oocytes competence for fertilization and embryo development in an *in vitro* environment.

In our first study (Chapter 3), the first experiment was designed to evaluate the effect of natriuretic peptide precursor type C (NPPC, 100-1000 nM), cyclic guanosine monophosphate (cGMP, 2-10 µM), cyclic adenosine 3, 5monophosphate (cAMP, 0.5-2 mM), nitric oxide (NO, 0.5-2 mM), and steroid hormones (testosterone, 500-1000 ng/ml ; progesterone, and estradiol, 100 ng/ml for each) to delay resumption of nuclear maturation during *in vitro* maturation of porcine cumulus oocyte complexes (COCs). Meiotic resumption was inhibited at an optimum concentration of 1000 nM for NPPC, 1mM for dbcAMP and NO, and 100 ng/ml for estradiol. The other substances (cGMP, testosterone, and progesterone) could not inhibit the meiotic resumption. Using the inhibitory substances, the second experiment evaluated the effect of delaying nuclear maturation for different times during IVM process (2, 6, 18, 22 hrs). Estradiol, NPPC, cAMP, or a combination of these substances were able to inhibit the meiotic resumption for 22 hrs. Three treatments (NPPC + estradiol (NE); cAMP (CA); NPPC + estradiol + cAMP (CANE)) were chosen to delay nuclear maturation (n= 450-500 COCs /group) and evaluate their effects on embryo development. Interestingly, blastocyst rate ranged from 6.0-8.7% and did not differ between the treatment groups or treatments to the control (9.4%, porcine follicular fluid in maturation medium, Pff). The selected treatment could be considered a base to develop a medium without animal source products like Pff.

As a part of the second experiment (Chapter 4), the effects of inhibitory substances (NE, CA, CANE) on molecular pathway and gene expression profile of in vitro matured pig oocytes was evaluated. Microarray technology was used to assess the genes expression profile associated with different treatments compared to Pff group. The results revealed that 120, 55, and 167 genes were differentially expressed in matured oocytes for NE, CA, or CANE, respectively. The data were analyzed using EASE program to classify genes into different ontology categories. While DAVID and PANTHER programs were used to identify the most significant molecular pathways induced by each treatment. The results showed CA treatments significantly up-regulated mitochondrial ribosomal protein L3 (MRPL3) and heat shock protein 90kDa alpha (HSP90AA) genes. Also, Janus kinase / signal transducer and activator of transcription (JAK/STAT) and Platelet-derived growth factor (PDGF) pathways were up-regulated. The NE treatments up-regulated several genes: MYC associated factor X (MAX), mitogen-activated protein kinase 9 (MAPK9), and v-rel reticuloendotheliosis viral oncogene homolog (REL) involved in cellular proliferation and the development of oocytes. While CANE was found to up-regulate IGF1 gene. The results of this study showed that using different substances to delay meiotic resumption would change the response of molecular pathways and genes expression of matured oocytes. However, the effects of these treatments groups on embryo development were equivalent to Pff group.

In our final study (Chapter 5), *in vitro* produced pig embryos were vitrified using two different devices: a closed device (French Straws (FS), n=127) or an open device (Open Pull Straw (OPS), n=115). After vitrification and warming, the embryos were graded based on their morphological quality and cultured for 48 hrs. The quality of embryos was also evaluated at 48 hrs of embryo culture. The results showed that almost 85% of the vitrified embryo were quality 1 after the warming procedure for both devices. However, the quality of embryos (Grade 1) slightly decreased in OPS compared to FS after 48 hrs of culture (FS=88, OPS=72). Vitrified-warmed embryos did not grow from morula to blastocyst, suggesting sub-optimal culture conditions or

cryo-damage affecting the development of embryos. These results indicated that using FS in a vitrification protocol can be used to preserve *in vitro* produced pig embryos on long-term basis. This thesis was demonstrated that delaying meiotic resumption of pig oocytes using NPPC+estradiol, cAMP or NPPC+estradiol+cAMP treatments can support embryo development by improving oocyte competence through different molecular effects. Also, these *in vitro* produced embryos could be stored in liquid nitrogen in a closed devices (French Straw).

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DEDICATION

I would like to dedicate this thesis to my wife, for her unyielding love…. without her support, I would not go progress.

Without you, I never would have tried

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CA	cAMP treatment
cAMP	Cyclic adenosine 3, 5monophosphate
CDK1	Cyclin-Dependent kinase 1
cGMP	Cyclic guanosine monophosphate
DAVID	Database for Annotation, Visualization and Integrated Discovery
E	Estardiol
EASE	Expression Analysis Systemic Explorer
Fig.	Figurer
FS	French Straws
FSH	Follicle-stimulating hormone
GJC	Gap junctional communication
GV	Germinal vesicle
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hrs	Hour(s)
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* embryo production
LH	Luteinizing hormone
LN2	Liquid nitrogen
MAPK	Mitogen-activated protein kinases
mg	Milligram
MI	Metaphase I
MII	Metaphase II
mL	Milliliter
mRNA	Messenger ribonucleic acid
NE	NPPC plus estrdiol

CANE	cAMP+ NPPC+ estradiol treatment
ng	Nanogram
NO	Nitric oxide
NPPC	Natriuretic peptide precursor type C
OPS	Open Pull Straw
PCR	Polymerase chain reaction
Pff	Porcine follicular fluid
PI	Propidium iodide
PKA	Protein kinase A
qPCR	Real-time polymerase chain reaction
RIN	RNA integrity number
ROS	Reactive oxygen species
vs	Versus
JAK	Janus kinase

CHAPTER 1

1.0 LITERATURE REVIEW

1.1 Introduction

Pork is the world's most consumed meat and accounts for over 36% of global meat intake [1]. However, the Food and Agriculture Organization of the United Nations (FAO) report (2013) indicates that 23% of worldwide pig breeds are at risk of extinction, and indeed 109 breeds are already extinct [2]. This fact highlights a significant threat to genetic diversity, especially given that most of current pork production comes from just a few domestic breeds [3]. For this reason, many countries, including Canada, have committed to protect the genetic diversity of different species by preserving their genetic material as sperm, oocytes, tissue, or embryos [4]. Thus, assisted reproductive technology, including *in vitro* embryo production, is being used as a tool to mature oocytes or produce embryos needed for preserving or improving animal breeds.

In vitro embryo production (IVP) has been widely used to generate a vast number of viable embryos for biotechnical manipulation or commercial purposes in farm animals [5]. The IVP technique has three independent steps: *In vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) [6]. The *in vitro* embryo production technique has been applied to different mammalian species to gain many advantages including: improving the genetics of the herd, controlling disease outbreaks, and preserving embryos for the long term (gene banking). Although IVP has been progressively developed over three decades [7], the efficiency of using this technique is still low in pig compared to the use of IVP in cattle or mouse [6]. In pigs, the IVP procedure still faces certain problems that have remained unsolved over the years, such as insufficient oocytes maturation, a high incidence of polyspermy (oocyte fertilized by several spermatozoa) and low embryo development [8, 9].

One of the main reasons for the low developmental competence of *in vitro*- produced embryos is the poor cytoplasmic maturation of oocytes during *in vitro* maturation (IVM) [6, 9]. To overcome this problem, temporary inhibition of meiotic resumption during the first stage of the IVM period (first 20-22 hours) could synchronize the progress of maturation and improve oocyte competence [10-12]. During meiotic arrest, protein synthesis continues in the oocytes [13] to accumulate factors in the cytoplasm to support embryo development, through communication with the cumulus cells. For this reason, many substances inhibiting meiotic resumption

(including dbcAMP, roscovitine and butyrolactone) have been used to improve cytoplasmic maturation [13-15]. The temporary inhibition of meiotic activity can be achieved by suppressing the activity of the maturation promoting factor (MPF) [16] or by elevating the the intercellular level of cyclic adenosine monophosphate (cAMP) [10]. This research focuses on improving cytoplasmic maturation during *in vitro* maturation of pig oocytes with the ultimate goal of producing a sufficient number of competent embryos.

1.2 Oocyte development and its competence

Oogenesis is a process of development of germ cells into primary oocytes, secondary oocytes and finally ovum [17]. This process starts when the primordial germ cells migrate from the yolk sac to gonads, where they multiply from a few thousand of cells to millions [18]. In pigs, germ cell meiotic division continues from day 13 of embryonic life until day 7 following birth [19]. Proliferating germ cells are called oogonia that differentiate into primary oocytes. Meiosis in pigs begins in oogonia during early embryonic life (Day 40) and stops around 35 days after birth when all oogonia arrest in the prophase of the first meiotic division (germinal vesicle, GV) [19, 20]. The transition from meiotic to a meiotic program is regulated by various factors and signals coming from the somatic cells around the oocyte [21]. The structure of the primary oocytes are enclosed by a single layer of flattened pre-granulosa cells, which the complex is referred to as primordial follicles [22]. When the granulosa cell appearance changes from flat to cuboidal, the primordial follicle becomes primary [18]. During secondary follicle development, the oocytes grow, and the number of granulosa cell layers increases. Finally, the antral follicle (tertiary follicle) is marked by the formation of fluid-filled cavity, called antrum [23]. Most of oocytes collected from follicles of a size between 3-8 mm can reach to metaphase II (MII) [24]. During folliculogenesis, size of pig oocytes grow from 20 to 160 µM [25], and many structural changes occur in the oocytes that make the oocytes competent (matured) for fertilization and embryo development [6, 19]. The maturation of oocytes is done on two levels: cytoplasmic and nuclear maturation. These two levels of maturation are explained in the following section.

1.2.1 Nuclear maturation

Nuclear maturation can be identified as the ability of the oocyte nucleus to develop from the GV stage to metaphase II of meiosis [26]. There is a positive relationship between the diameter of the

follicle and the meiotic activity; follicles of diameter < 3mm are generally incompetent to progress in pigs [27].

In general, oocytes from a majority of mammalian are arrested after birth in prophase I of meiosis I until puberty [28, 29]. However, the oocytes resume meiosis spontaneously when they are removed from the follicles and introduce in an *in vitro* envrionment [30]. The nuclear maturation process has been classified based on the morphological changes that take place in the chromosome during meiosis. Before meiotic resumption, oocytes contain diffuse chromosomes that are enclosed within the intact nuclear structural cell that is referred to as the germinal vesicle (GV). At meiotic resumption, the chromosome begins to condense and GV breaks down (GVBD); the nuclear membrane almost disappears. GVBD leads to diakinesis where the chromatin undergoes condensation into either a single lump or smaller discrete fragments [31, 32].

As maturation progresses, metaphase I starts to align with the paired homologous in the middle of the forming meiotic spindle. As meiosis proceeds to anaphase I (ana I), the bivalents of the chromosomes separate and start to move to opposite poles of the cell due to the action of the spindle. The separation (telophase I) is resulting in two diploid daughter cells, namely, the first polar body and the oocyte. By metaphase II, the polar body chromosomes begin degradation while the chromosomes of the oocyte are arranged on the meiotic spindle [33, 34]. At this stage, the meiotic division I is finished, and the oocytes are ready for fertilization. After sperm cells penetrates the zona pellucida and one sperm cell enter into the oocyte, meiosis II resumes by a transaction from MII to ana II, leading to a release of the second polar body. The sperm nucleus condenses, and two haploid nuclei are formed (male and female pronuclei) in the fertilized oocyte [26].

In vivo, it was found that injecting pigs with hCG led to GVBD in about 18 hrs after gonadotropin exposure and meiosis was completed (MII) by 36-40 hrs [35]. *In vitro*, the nuclear maturation can be achieved (MII) in the pig oocyte in a time ranging from 36-48 hrs [36, 37]. Although the rate of nuclear maturation *in vitro* is high and almost similar to that *in vivo*, the number of oocytes that develop to the blastocyte stage are often small (< 30%) in pigs [36, 37]. This fact indicates that oocyte competence during *in vitro* maturation relates more to cytoplasmic maturation than to nuclear maturation.

1.2.2 Cytoplasmic maturation

Cytoplasmic maturation can refer to all molecular and ultrastructural changes in oocyte cytoplasm supporting the developmental competence of the oocytes [38-40]. During cytoplasmic maturtion, oocytes accumulate mRNA and proteins [40, 41]. Proteins that are expressed from mRNA have a significant role in oocytes maturation, fertilization and early embryonic development [42, 43]. Cytoplasmic maturation can be considered as significant factor that affect matured oocyte to undergo normal fertilization and blastocyst formation. Therefore, the level of embryo development (cleavage and blastocyst rate) can reflect the cytoplasmic maturation condition indirectly.

The Intracellular glutathione (GSH) plays an essential role in protecting cells against oxidative stress and maintaining intracellular redox status [44]. Also, GSH concentration increases during oocyte maturation and is involved in male pronucleus (MPN) formation that coincides with oocyte activation [45]. *In vitro*, cysteine and cysteamine (GSH precursors) have been used in maturation media and have been shown to improve the embryo developmental rate of a pig or bovine oocytes [46, 47]. Cumulus cells were found to participate critically in the GSH content of the oocyte [48] by supplying the cysteine to oocytes or produce and transport GSH to oocytes [8]. Supporting this role for cumulus cells, maturation of denuded oocytes did not improve their developmental competence [49, 50]. The GSH content of oocytes is used to evaluate cytoplasmic maturation [6, 51].

Other important cytoplasmic maturation changes are cortical granule translocation and the intracellular calcium level [52]. During oocyte maturation, cortical granules migrate to the cortex of the oocytes and attach beneath the oolemma. After fertilization, the content of cortical granules are released into the perivitelline space to block polyspermy by modifying the oocytes. extracellular matrices [53]. This secretory process is calcium dependent and known as a cortical reaction [54]. The amount of calcium stored in the oocytes increases significantly in matured oocytes compared with those that are immature [55], showing the importance of calcium for oocytes activation.

Mitochondria are other cytoplasmic organelles

playing a key role in different cytoplasmic processes during oocyte maturation, as they are the main supply of energy [56, 57]. Mitochondria synthesize adenosine triphosphate (ATP). They are considered an internal source of Ca^{2+}. The low mitochondrial content has been linked to the

failure of fertilization and abnormal embryogenesis [58]. During oocyte maturation, the pattern of mitochondrial distribution and its metabolic activity undergoes several changes [59]. Pig mitochondrial transfer from the peripheral part of the oocyte to the inner ooplasm of the cell [59, 60]. It was also found that ATP content increased significantly during *in vitro* maturation [61] and had a similar amount in those pig oocytes that were matured either *in vivo* or *in vitro* [56]. The endoplasmic reticulum (ER) also undergoes significant changes during oocyte maturation. The membranes of ER have many known functions, including: lipid metabolism, protein folding and degradation, compartmentalization of the nucleus, and membrane synthesis [62]. Further, regulating the intracellular releases of calcium is an important structural change in the ER during maturation and has an essential role in oocyte activation [63, 64]. In porcine oocytes, the ER distributes throughout the cytoplasm from the GV to the MI stage of oocyte maturation. However, at the MII stage, the ER produces uniquely large clusters at the animal pole of the cytoplasm, around the first polar body [65].

1.2.3 Control of oocyte meiotic arrest

Fully grown oocytes are maintained in meiotic arrest (Prophase I) within the antral follicle until meiosis resume when the LH surge occurs. However, cumulus oocyte complexes (COCs) resume meiosis spontaneously when COCs are removed from follicles and cultured [30]. This observation indicates that the meiotic arrest process is controlled by several factors that exist in follicular fluid and are secreted from mural granulosa cell origin [66]. These factors were shown to act on cumulus cells and were blocked by LH [66, 67]. Many studies have identified intracellular and paracrine interaction factors between oocyte and somatic cells that are involved in the meiotic arrest [68-71]. One of the key components in maintaining the meiotic arrest process is cyclic adenosine monophosphate (cAMP) level in the oocytes [68, 72]. The high cAMP level suppresses the activity of the CDK/cyclin B protein complex, also known as maturation promoting factor (MPF) [71]. However, when the MPF is activated, meiosis resumption occurs. In the following section, most of the components that are involved in the meiotic arrest are explained.

1.2.3.1 cAMP:

This secondary messenger plays a regulatory role in the meiotic oocyte cycle. The cAMP is synthesized by adenylate cyclase (ADCY) under the control of gonadotropins (FSH and LH) [73]. Also, cAMP is considered as a regulator for gap junctional communication [74]. Decreasing the level of cAMP is a signal to resume meiosis. There are two mechanisms that elevate the cAMP level and maintain the meiotic arrest. For a long time, it was believed that the majority of intra-oocyte cAMP is generated by granulosa cells and diffused into the oocytes through gap junctions from the cumulus cells [75]. However, some studies in rodent and human have indicated that the oocytes themselves can generate cAMP to maintain meiotic arrest by G-protein coupled receptors (GPR3 and/or GPR12) and stimulate ADCY [69, 76, 77]. It was observed that mice oocytes having a deficiency in ADCY or GPR3-knockout resumed meiosis spontaneously [78, 79]. Also, when porcine oocytes were cultured with forskolin, cAMP production increased by stimulation of ADCY production [80]. These results indicate that the GPR/ADCY pathway in oocytes could be an essential for cAMP production [71]. In pigs, GPR3 is found to be essential for maintenance of meiotic arrest in oocytes, through the pathways involved in the regulation of cAMP [81]. A second regulator for cAMP level in oocytes is a phosphodiesterase 3A (PED3A) enzyme that is involved in the hydrolysis of cAMP to 5-AMP [82]. PED3A has been located only in oocytes [69]. Suppressing PED3A activity is required to prevent cAMP degradation and to maintain a high level of cAMP in the oocytes. Cyclic guanosine monophosphate (cGMP) is responsible for inhibiting PED3A activity [83, 84]. The cGMP is synthesized in cumulus cells and it is moved to the oocytes through gap junctions [84, 85]. It has also been reported that PED3A is downstream of GPR3 in the regulation of the cAMP level in the oocytes [86]. The concentration of cAMP in oocytes is controlled by its degradation (PED activity), rather than by endogenous synthesis [87]. In *GPR3* knockout mice, it was found that the spontaneous progression of meiosis was blocked by an injection of GPR3 RNA into preantral follicle-enclosed oocytes, followed by culture to the early antral stage [79].
Various chemicals have been used to elevate the intracellular level of cAMP in maturation media: dibutyl cyclic adenosine monophosphate (dbcAMP) and cycloheximide [88, 89]. Most of these chemicals increase the level of cAMP depend on two mechanisms: stimulating the ADCY enzyme or inhibiting the PDE3A enzyme. More details will be discussed in section 1.5.1 for the *in vitro* method to increase the intracellular level of cAMP.

1.2.4.1.1 How do high cAMP concentrations in the oocytes prevent meiotic resumption?

The major component that regulates the activity of the maturation promoting factor (MPF) in oocyte is protein kinase A (PKA). A study in mouse oocytes observed the inhibition of PKA-induced GVBD in oocytes [79]. Therefore, PKA is considered the mediator of cAMP activity in oocytes. However, the effect of PKA is not directly on MPF (CDK1/cyclin B complex), but it regulates through the activity of Wee1/myclin transcription factor 1 (Wee1B/MYT1 kinase) and cell division cycle (Cdc25B) phosphatase [90]. The high level of cAMP in oocyte stimulates PKA, which activates Wee1B to produce phosphorylated CDK1 leading to inhibition of CDK1 [71]. As a result, inactive CDK1 inhibit the MPF. It was found that Wee1B mRNA expression increased in oocytes during the GV stage and it down-regulated in early meiosis resumption in both *in vivo* and *in vitro* maturation [91, 92]. On the other hand, high PKA level inactivated Cdc25B (diphosphatase CDK1) by phosphorylation serine residue at position 321 [93]. When Cdc25B is inactivated, it is transferred from the nucleus to cytoplasm, away from the phosphorylation activity of CDK1 in nucleus [94]. Therefore, the MPF complex becomes inactive and the oocyte is maintained at the GV stage. At the time of meiotic resumption, the PKA activity decreased leading to activation of Cdc25B, removing the inhibitory phosphorylation caused by Wee1B. Then, CDK1 is activated which stimulates the MPF (Fig.1.1; the description is based on a review from Liu *et. al.* [82]).

1.2.3.2 Natriuretic peptide precursor Type C (NPPC):

The natriuretic peptide system family has three types of structurally related forms: The natriuretic peptide precursor type A (NPPA), natriuretic peptide precursor type B (NPPB) and natriuretic peptide precursor type C (NPPC or CNP) [95]. NPPA and NPPB are mainly produced in atrial and ventricular cardiomyocytes, respectively [96]. NPPC is generated by mural granulosa cells, and its cognate receptor natriuretic peptide receptor 2 (NPR2) is located primarily in cumulus cells [97]. It has been shown that NPPC increases the intracellular cAMP concentration in mice COCs by promoting cyclic guanosine monophosphate (cGMP) production in the cumulus cells through its receptor NPR2 [97]. The cGMP is transferred to the oocytes via the gap junctions to inhibit the activity of PDE3A in the oocytes [69, 98, 99].

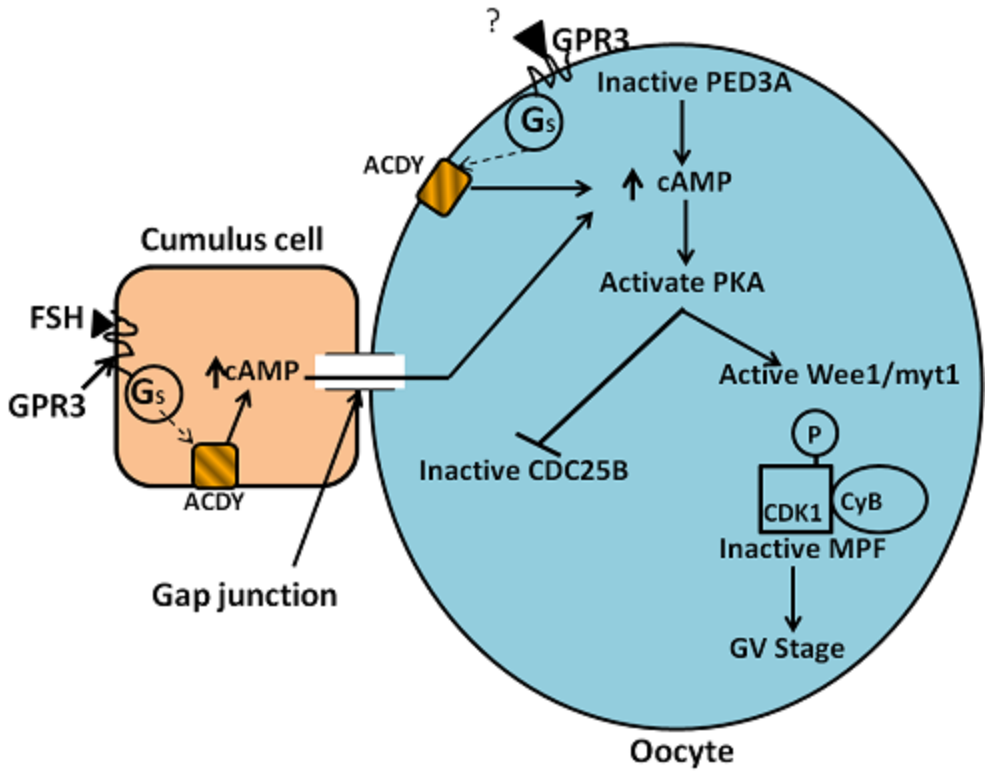

Fig. 1.1 Model of meiotic arrest in the porcine oocyte. Intra-oocyte cAMP is produced by G-protein coupled receptor 3 (GPR3) that activate adenylyl cyclase (ADCY) endogenous to the oocyte. The cGMP is transferred from cumulus cells inhibit phosphodiesterase (PDE) 3A activity and cAMP hydrolysis, maintaining cAMP levels high. The cAMP-dependent protein kinase regulates the activity of maturation-promoting factor (MPF) by the phosphatase cell division cycle 25 (CDC25) and the kinases Wee1 and myelin transcription factor 1 (Myt1): CDC25 dephosphorylates cyclin-dependent kinase 1 (CDK1), whereas Wee1 and Myt1 phosphorylate CDK1. High cAMP levels result in the phosphorylation of CDK1 and inactivation of the MPF complex (CDK1–cyclin B (CyB)) leading to maintaining the oocyte at the germinal vesicle stage. (Adapted from Liu *et. al.*[82])

By blocking the activity of PDE3A via a high level of cGMP, the concentration of cAMP in the oocytes increased (Fig 1.2), leading to the arrest of meiotic resumption. In a study on mouse oocytes, adding NPPC (100 nM) to the maturation media inhibited resumption of meiosis (GVBD rate 98% in control vs <10% in the treatment group) [97]. The expression of NPPC in mural granulosa cells has been found to be stimulated by FSH receptors activated by equine chorionic gonadotropin (eCG) [100, 101]. In addition, eCG treatments increased NPR2

expression in cumulus cells [102]. *In vitro*, FSH did not show the same *in vivo* effect on NPR2 mRNA expression in the cumulus cells [100]. This observation may indicate that the FSH effect is indirect on NPR2 expression [82]. It was found that using NPPC plus estradiol or testosterone inhibited the meiotic resumption significantly in mouse oocytes compared to NPPC alone [100]. Interestingly, the same study showed that testosterone and estradiol increased the mRNA expression of NPR2. In this specific pathway, the oocytes may play an important role by producing many oocyte factors, such as growth differentiation factor 9 (GDF9) and bone morphogenetic portion 15 (BMP15) or fibroblast growth factors 8 (FGF8) [69, 97]. These oocytes derived paracrine factors (ODPF) increased the expression of NPPC and/or NPR2 via the stimulation of estradiol production [103, 104]. This pathway may demonstrate a type of control mechanism for the oocytes to maintain the meiotic arrest (Fig. 1.2). Therefore, NPPC and its receptors (NPR2) are thought to play a critical role in the maintenance of the meiotic arrest, producing a high level of cGMP and increasing the cAMP level in oocytes.

1.2.3.3 Nitric oxide (NO):

Nitric oxide is a short-lived messenger that involves in a variety of physiological functions including a regulatory role in the nervous, immune, and cardiovascular system [105-107]. Also, NO plays a critical role in many reproductive events such as ovulation, oocyte meiotic maturation, and fertilization [108, 109]. The nitric oxide synthase (NOS) synthesizes NO in cells using L-arginine and oxygen [110]. There are three forms of NOS: Neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) [111]. The iNOS and eNOS are found expressed in granulosa and theca cells [112, 113]. On the follicle, NO is essential for maintaining and increasing the rate of ovarian blood flow during the pre-ovulatory period [112]. Also, NO has a significant function before and during embryonic development [114].

To study nitric oxide (NO) cellular responses, nitric oxide donors such as S-Nitroso-N-acetylpenicillamine and S-Nitrosoglutathione have been used to stimulate physiological conditions resulting in the donation of NO [115]. Several studies have shown that NO donors inhibited rodent oocytes meiotic resumption [116, 117]. However, a NO donor can have the opposite action depending on the dosages [108]. In pig, culturing oocytes with a NOS inhibitor and NO donor inhibited the meiotic resumption of oocytes [118] or blocked meiotic maturation [119]. Interestingly, it was found that iNOS expression in granulosa cells decreased after eCG

injection, which indicated that the level of NO metabolism also decreases after eCG injection [120]. Another study found that using an iNOS inhibitor (L-NAME) decreased cGMP production in granulosa cells [121, 122].

In general, the high level of NO can cause an inhibitory effect on meiotic resumption of oocyte in mice [123], rats [124] and pigs [118, 125]. The production of NO can be regulated by estradiol. A study of porcine oocytes found that adding estradiol to maturation media promoted the synthesis of NO without any increases in eNOS expression [126]. Soluble guanylate cyclase (sGC) is the main receptor for NO in cumulus cells. The sGC plays a significant role in the production of the cellular messenger molecule, cGMP [127]. Therefore, nitric oxide can be an important component in meiotic arrest as it increases the cGMP level and subsequently increases the level of cAMP to prevent meiotic resumption.

1.2.3.4 Cyclic guanosine monophosphate (cGMP):

This second messenger plays an activation role of intracellular protein kinases [128]. Increase of cGMP level in oocytes is associated with a delay of meiotic resumption [129], suggesting that cGMP have an inhibitory effect of nuclear maturation. Cumulus cells produce cGMP, and it is transferred through gap junctions to the oocytes [71, 89]. Increasing the level of cGMP-inhibited the PDE3A in the oocytes [130]. This inhibition of PDE3A would prevent cAMP degradation therefor keeping intracellular cAMP high in the oocytes to maintain the meiotic arrest. In *vitro*, porcine cumulus oocytes treated with cGMP analogs (0.1-1mM) during maturation inhibited meiotic resumption and increased significantly the intracellular level of cGMP [131]. Another study on porcine oocytes found that adding 5 mM of cGMP was enough to inhibit the meiotic resumption [132]. Also, it was found that treatment of the COCs with LH decreased the level of cGMP via activation of the EGF network [98]. These different observations demonstrated the regulatory function of cGMP on the meiotic arrest.

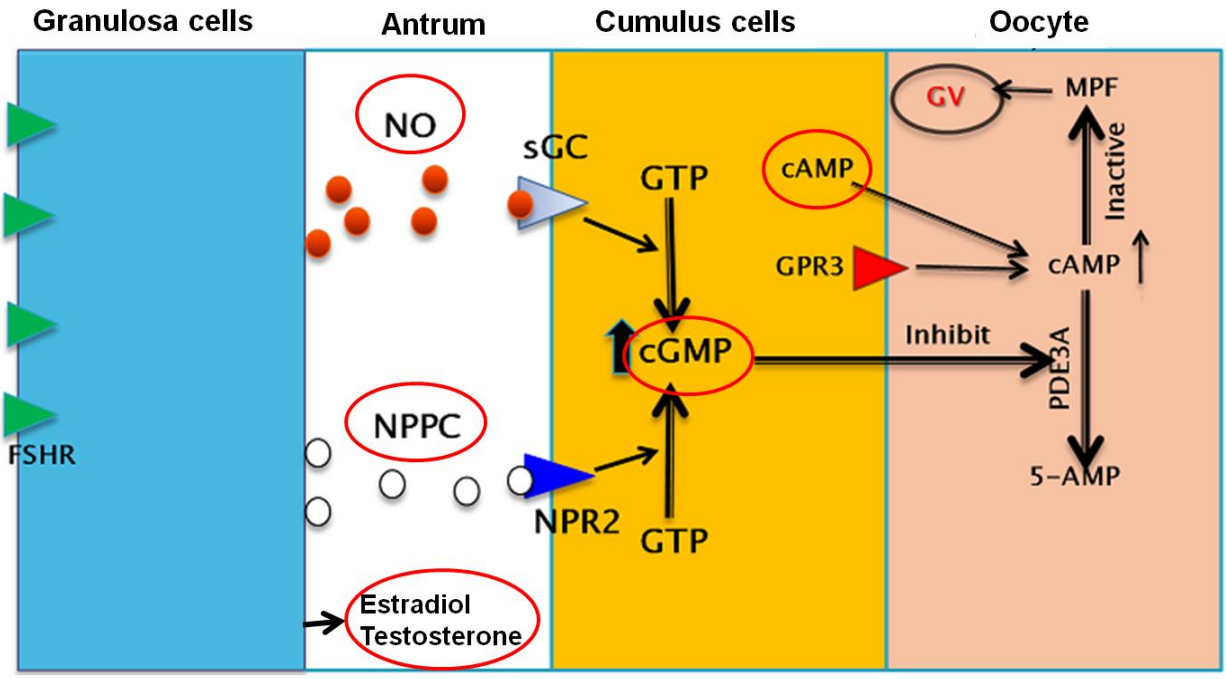

Fig.1.2 Mechanism of molecular pathways of nitric oxide (NO) and natriuretic peptide protein type C (NPPC) in a cumulus-oocyte-complex maintaining meiotic arrest. NPR2: natriuretic peptide receptor 2, sGC: Soluble guanylate cyclase, MPF: Maturation promoting factor, GTP: Guanosine-5'-triphosphate, GPR3: G- protein coupled receptors, GV: Germinal vesicle, PDE3A: Phosphodiesterases 3A, AMP:Adenosine monophosphate and ODPF: oocytes derived from paracrine factors.

1.2.3.5 Hormones (testosterone, estradiol, and progesterone):

Steroid hormones were found in follicular fluid during oocyte maturation at different levels. These hormones play a critical role in oocyte modifications regarding nuclear and cytoplasmic level [133-135]. In a study on bovine COCs, adding aminoglutethimide to maturation media to block steroid hormone secretion negatively affected the cumulus cell expansion [135]. Steroid hormones (testosterone, estradiol, and progesterone) were found to be involved in different pathways of meiotic arrest. Estradiol increased production of many factors that inhibit meiotic resumption such as NPR2, NO and NPPC [100, 102, 136]. It is reported that the mRNA level of NPR2 increased when immature rats are treated with synthetic estrogen diethylstilbestrol [137]. Estradiol treatment also promotes expression of NPR2 at the mRNA level of mouse cumulus cells. The elevation in NPR2 expression leads to an increase of cGMP concentration in response

to NPPC and maintained meiotic arrest with NPPC treatment [100]. In another study, when porcine COCs were treated with estradiol (10 nM), NO production increased in comparison to progesterone and testosterone [126]. Testosterone treatment on mouse cumulus cells also increased NPR2 expression and meiotic arrest *in vitro* [100]. The testosterone action on NPR2 is likely due to the aromatization of testosterone to estrogen, rather than androgen action, supporting the fact that using dihydrotestosterone (non-aromatizable androgen) did not stimulate the expression of NPR2 [100]. Another study found that treatment of mouse oocytes during *in vitro* maturation with a high testosterone concentration (≥ 40 μM) inhibited meiotic resumption, while a low testosterone concentration could not prevent the inhibition of meiosis [138]. Testosterone was also found to increase the inhibitory effect of dbcAMP on meiotic resumption in porcine oocytes [139].

1.3 In vitro embryo production (IVP)

In vitro embryo production is a procedure consisting of three steps that allow for the production of embryos. In the first step, immature oocytes undergo a process called *in vitro* maturation (IVM). During this stage, the oocytes undergo many changes at a cytoplasmic and nuclear level to be ready for *in vitro* fertilization (IVF). In this step, the matured oocytes are incubated with spermatozoa to generate the zygote. The third process is *in vitro* culture (IVC). In this stage, presumptive zygotes are cultured in media and supplied with the necessary nutrients for normal embryo growth until the blastocyst formation [5].

1.3.1 Importance of in vitro production of embryos

Large scale *in vitro* production of porcine embryo can be derived from a significant number of oocytes from slaughterhouse derived ovaries. Therefore, IVP can produce a large number of viable embryos at a low cost compared to surgical embryo collection [47]. Embryos can be used for research in reproductive physiology including micromanipulation of embryos to provide genetically modified swine as potential donors of tissues and organs [5]. The IVP of pig embryos can be utilized to address various economic traits, such as growth rate and food efficiency, and to increase productive efficiency and the rate of genetic gain [140]. In addition, the availability of embryos can enhance the biosecurity of the swine industry by decreasing the use of live replacement animals. The high cost and the risk of disease transmission that is associated with

live animals also make the use of the embryos favorable [141]. However, the high cost of surgical transfer of embryos has limited commercial applications. Successful cryopreservation of pig blastocysts [142] and embryo transfer of rare pigs breeds [143] highlights the importance of IVP in gene banking of a rare or valuable breed. Cryopreservation of pig embryos could be considered as a protective measure against disease outbreak. Genetic material can be stored for lengthy periods to protect the breed from extinction and to maintain genetic diversity. IVP can also be used to stimulate research on factors controlling embryonic development and to identify factors to prevent significant economic losses due to the high rate of early embryonic mortality [144].

1.3.2 In vitro maturation (IVM)

Oocytes collected from ovaries obtained from slaughtered animals are immature regarding nuclear and cytoplasmic maturation. During the IVM process, the oocytes are exposed to specific media for 40-44 hrs to resume the meiosis until metaphase II (MII) and to prepare them for fertilization [6]. During this period, the cytoplasm undertakes many structural changes that are critical for subsequent embryo development. The maturation medium contains many supplementations (such as hormones, growth factors, and follicular fluid [145]) to promote cytoplasmic and nuclear maturation.

Many morphological criteria need to be considered in selecting follicles, COCs or oocytes. There is a positive relationship between follicle size and oocytes developmental competence [24]. Oocytes that are collected from the small follicles (< 3 mm) are less competent to support embryo development, while the oocytes of medium follicle size (3-8 mm) have increased likelihood [146]. Most IVM protocols recommend harvesting follicles from 3-6 mm in size for pig ovary [147]. Cumulus cell layers surrounding the oocytes represent another important parameter that correlates with oocytes competence. As previously discussed, cumulus cells supply the oocytes with many signals and molecules required for oocytes maturation [148]. It is recommended to select COCs with at least three layers of cumulus cells [8]; thicker layers could reflect more competent oocytes [149]. At the end of IVM, cumulus cell expansion can be used as an indicator of full maturation of oocytes [148].

Different maturation media have been used in various laboratories such as TCM-199 [37], NCSU-23 [150], and POM [151]. Most of these media aim to have same of *in vivo* conditions as

much as possible. In general, most maturation media are supplemented with gonadotropins (LH, FSH). These hormones are required to stimulate meiotic resumption and other oocytes functions. FSH activates cumulus expansion and supports nuclear and cytoplasmic maturation [152]. It also induces the expression of factors such as hyaluronic acid and cAMP that control many functions in oocytes [153]. The actions of FSH mainly takes place indirectly on oocytes via granulosa cells [154]. The *in vitro* role of LH is controversial, as some reports indicating that LH receptors in cumulus cells were not present in cattle [155, 156]. However, LH receptor expression in cumulus cells in pig was reported [157-159]. As LH plays an important role in oocyte maturation, most the IVM procedures include this gonadotrophin in their maturation media. Growth factors are also added to IVM media, such as epidermal growth factor (EGF) [160], and essential and nonessential amino acids [161]. The main beneficial effect of EGF is activation of the synthesis of intracellular glutathione supporting embryo development and protecting DNA [162].

1.3.3 In vitro fertilization (IVF)

In vitro fertilization is a process that incubates matured oocytes and spermatozoa to allow fertilization. When spermatozoa are exposed to culture media, many morphological and biochemical changes occur in the sperm to prepare them for fertilization, defined as capacitation. However, successful IVF depends more on the preparation procedure for spermatozoa [163], oocytes and the interactions of sperm-oocyte. Co-culture of porcine oocytes with oviductal epithelial cells have also elevated the percentage of monospermic oocytes [164]. These results indicated that a factor(s) from the oviductal fluid is important for the maintenance of monospermic fertilization penetration. Therefore, many aspects of the IVF protocol were considered regarding polyspermy and the different protocols were modified to minimize such effects.

Many elements are integral to a successful IVF procedure including sperm-oocyte incubation time, IVF media conditions, and sperm concentration. A study evaluated sperm-oocyte incubation periods ranging from 3 to 12 hrs; they found that 6 hrs was the optimal incubation time based on a maximal penetration rate (81%) with polyspermy around 39% [165]. As incubation time increased, the number of polyspermic presumptive zygotes increased with no change in penetration rate [166]. Therefore, it is recommended to incubate spermatozoa with oocytes for 6 hrs [165, 167]. However, another study found that 6 hrs of incubation with sperm can increase the number of sperm cells into the oocyte compared to 3 hrs incubation [168].

Incubation of sperm with oocytes over a short time (10, 30, 60 min) compared to 6 hrs did not improve embryo development or reduced the rate of polyspermy [169].

Different types of fertilization media mimicking an *in vivo* system have been used in porcine IVF such as: modified tris buffer medium (mTBM), Tyrode's medium (TALP), and IVF medium-199 (IVFm-199) [5]. A fertilization medium is needed to support the physiological functions of both oocytes and spermatozoa. Bovine serum albumin is an important component in IVF media to induce capacitation and to regulate penetration of the sperm in the oocyte [165, 170]. However, it could increase the mean number of sperm per oocytes [165]. Extracellular calcium is also required in IVF media because of its role in capacitation and maximal acrosomal exocytosis [171]. Caffeine is another common component used in IVF media. It has a role in sperm capacitation and a positive effect on embryo development [172].

1.3.4 In vitro culture (IVC)

The final step for *in vitro* embryos production is *in vitro* culture. The presumptive zygotes grow in an environment (5% O_2, 5% CO_2, and 90% N_2) [173] that mimic *in vivo* conditions for embryo development. The culture medium contains many supplements that support embryo development. Most culture media contain different types of salts at a low concentration [174] and energy resources such as glucose [173]. Increasing NaCl concentration (94.88 vs. 68.49 mM) in a culture media impaired embryo development compared to media with a lower NaCl concentration (respectively 20 and 10 vs.13% and 73% for morula and blastocyst, respectively) [174]. The pattern of energy resource available in a culture media can make differences in the results of embryo development. In a study, culture media was supplied with pyruvate and lactate for the first two days and then replaced with glucose for another four days [173]. The results of this study improved the blastocyst rate in an *in vitro* porcine system. Another study found that increasing the time using pyruvate and lactate from 48 to 58 hrs before replacing the media with glucose was more beneficial for blastocyst formation (20.6 vs 31.3% reparctively) [175]. Using glucose during the first two days of IVC was found to generate reactive oxygen species (ROS), which could induce apoptosis and provoked adverse effects on embryo development [176]. The addition of amino acids to culture media also improved embryo development in pig [177]. Taurine and/or hypotaurine into culture media has been shown to significantly enhancing the development of porcine embryos [178]. Also, supplementation of culture medium with oviductal

fluid supports embryo development [179]. Since the availability of oviductal fluid is limited in general routine embryo culture, most labs use BSA as a protein supplement [150], and to maintain osmotic balance and stable PH [180]. BSA has been shown to improve the development of porcine embryos [181]. Adding insulin or leptin to a could be beneficial for the embryo development during IVC. Lee *et al.* [182] reported that supplementing culture media with insulin to a culture media improved the blastocyst formation rate (34.1 vs. 23.1% in control). The blastocyst formation rate was also significantly elevated (two fold) when insulin was used during IVM and IVC (40.4% compared to 21.2% for untreated group). Using leptin (100 ng/ml) in culture media significantly increased the rate of blastocyst formation compared with other leptin concentrations or control (no supplement) [183].

Different types of culture media have been used for porcine embryonic culture, including modified Chatot, Ziomek, Bavister (CZB) [184], Beltsville embryo culture medium (BECM)-3[185], and PZM-5 [186]. One of the most popular culture media that has been used in porcine IVC is NCSU-23 [5]. This media was developed by North Carolina State University [150] and NCSU-23 was designed to meet the metabolism and nutrient requirements of pig embryos. A combination of NCSU-23with BSA was found to enhance blastocyst formation compared to other culture media [187]. Although NCSU-23 is the most commonly used media, some studies indicated that porcine zygote medium 5 (PZM-5) is more efficient in supporting the embryo development [151, 161]. PZM-5 is based on oviductal pig fluid compenents [186]. In summary, the success of IVC depends on the efficiency of the IVM/IVF process and its environment to culture embryos.

1.4 Challenges in IVP of pig embryos

Although the IVP technique has been progressively developed over three decades, the efficiency of IVP in pigs is still low compared to IVP in cattle or mouse [7]. In pigs, the IVP procedure still faces many problems that have remained unsolved over the years, such as insufficient cytoplasmic maturation, a high incidence of polyspermy and low embryo development. These problems are considered to be the main factors decreasing the efficiency of IVP in pigs [6] and these issues will be described briefly in the following sections.

1.4.1 Problems with in vitro maturation (IVM)

Having sufficient nuclear and cytoplasmic maturation of the oocytes is key to successful fertilization and embryonic development [188]. Nuclear maturation represents all changes at the chromosomal level, from the resumption of meiosis to reach MII stage. Generally, oocytes are collected from ovaries of slaughtered animals of different ages, different estrous cycle stages, and with variable follicular sizes. Therefore, these oocytes have different developmental stages. This situation creates heterogeneity in the level of nuclear and cytoplasmic maturation among the collected oocytes, and some oocytes could start meiosis earlier than others during IVM [189]. The variation of *in vitro* development of oocytes may reflect an imbalance of nuclear and cytoplasmic maturation rates within the cell [190]. Interestingly, the rates of *in vitro* and *in vivo* nuclear maturation did not differ [31]. In this study, oocytes were collected from gilts (*in vivo*) to evaluate the change of GVBD at particular times after hormone treatment (PMSG and HCG); while *in vitro*, oocytes were collected from the same group of gilts and cultured in the medium. The interval time to evaluate GVBD in oocytes *in vitro* was similar to *in vivo* group. In general, most IVM media that used to mature pig oocytes, such as NCSU-23, has ability to support oocytes reaching final nuclear maturation (MII) [191]. Consequently, it is believed that cytoplasmic maturation is the main factor of variation in the maturation of oocytes [6]. Low level of cytoplasmic maturation of oocytes negatively affects the accumulation of mRNA, proteins, substrates and required nutrients to support embryonic development [192]. One study found that insufficient cytoplasmic maturation is partially responsible for low MPN formation and blastocyst formation [9].

Cumulus cells play a crucial role in cytoplasmic maturation through providing many factors that are needed for cytoplasmic reorganization [8]. During cytoplasmic maturation, different components of the oocyte (such as mitochondria, golgi apparatus, cortical granules and endoplasmic reticulum) undergo structural changes [62]. It was hypothesized that oocytes accumulate sufficient amount of stable mRNA that play an important role in final molecular maturation [193]. Stored mRNA and translation at specific sites of the cytoplasm was found involved in many maturation process such as cell motility and development mechanisms that play role in oocyte competence [42]. These mRNAs play a significant role in different synthesis types of proteins involved in subsequence fertilization and embryonic development. These proteins are stored until the activation of the embryonic genome [194]. Also, many factors can

affect the quality of oocytes during the IVM process. The age of the slaughtered pigs is a significant factor. Many studies have shown that oocytes derived from pre-pubertal donors have a lower blastocyst development rate than adult donors [195]. The number of layers of cumulus cells surrounding the oocytes and the size of the follicle is also essential for supporting the maturation during IVM. Oocytes surrounded with > 3 layers of cumulus cells in IVM have higher cleavage and blastocyst rates compared to oocytes with < 3 layers [196]. Furthermore, the quantity of lipids in the cytoplasm during IVM plays a significant role in normal embryo development and successful embryo cryopreservation [11]. Pig oocytes contain a high level of lipids [197]. During IVM, the inhibition of fatty acid oxidation in the pig oocyte was found to impair embryo development, which indicated the importance of lipid metabolism [198]. To summarize, the main problem for IVM is related to poor cytoplasmic maturation that negatively affects embryo development in later stages.

1.4.2 Problems with in vitro fertilization (IVF)

The most challenging obstacle in IVF of porcine oocytes is the high incidence of polyspermy [199]. This problem can be caused by insufficient fertilization conditions and/or inadequate maturation of the oocyte leading to embryonic death [200, 201]. The incidence of polyspermy can reach 30-40% *in vivo* for matured oocytes and this rate increases to 65% with *in vitro* matured oocytes [199]. Many factors affect the incidence of polyspermy during the IVF process, including the degree of maturation of the oocyte [202], and the quality and the quantity of semen used in IVF [203].

It is believed that the high incidence of polyspermy can increase when oocytes do not reach an adequate level of competence, leading to delaying the release of cortical granules [204]. It has been shown that the membrane of the oocyte changes during the maturation process [205]. The zona blockage (to prevent extra sperm from penetrating the zona pellucida) becomes slower, allowing for more sperm to reach the oocyte [206]. Furthermore, the morphological appearance of zona pellucida of oocytes derived from IVM is different compared to ovulated oocytes [166]. The diameter and the thickness of the zona pellucida are bigger for *in vivo* matured oocytes compared to *in vitro* matured oocytes [207]. These observations indicate that the maturation associated with zona pellucida is not fully achieved in IVM oocytes [206], which could lead to a high rate of polyspermy. Furthermore, some additives to the IVF media can increase the rate of

polyspermy. Culturing porcine oocytes and spermatozoa in media containing caffeine increase the rate of polyspermy [208]. Also, it was found that the acrosome reaction occur faster, and polyspermy rate was higher in mTBM compared to TALP or IVFm-199 [209]. The penetration and cleavage rates were lower with mTBM than other media [209]. Another observation is that the ability of sperm cells to penetrate oocyte can change according to the boar [210]. In summary, polyspermy is the major issue associated with IVF.

1.4.3 Problems with in vitro culture (IVC)

Many obstacles that exist in the IVM and IVF processes are reflected in embryo developmental competence in terms of the blastocyst formation rate and cell number [6]. During the IVC period, the DNA fragmentation rate of blastocysts and apoptosis of cells are increased [211]. It was found that embryos that grown in IVC media had a lower number of nuclei and cell division compare to embryo growing *in vivo* conditions [187, 212]. Low cell numbers in blastocyst were associated with an increased embryonic mortality and decreased pregnancy rate [213]. Levels of supplements in the culture media can also critically affect embryo development. For example, Beckman and Day [174] observed an increase of embryo development (84% morula and blastocyst) when the NaCl concentration was higher in the culture media compared to lower concentrations (3% morula and blastocyst). Culture media can alter embryo development potential [214], the ultrastructural morphology [215], and transcript abundance [216]. In summary, IVC faces problems due to obstacles encountered in previous stages (IVM and IVF).

1.5 Importance of delaying meiotic resumption during *in vitro* maturation

Poor cytoplasmic maturation is one of the biggest obstacles to improving the results of IVP of porcine embryos. In bovine COCs, a study found that the breakdown of gap junctions occurs earlier *in vitro* compared to *in vivo* conditions (respectively 3 hrs vs. 9-12 hrs after the peak of LH) [217]. These results indicated that the time available for oocytes to accumulate many important factors and nutrients [8] could be less in *in vitro* compared to *in vivo* conditions. The gap-junctional communication between oocytes and cumulus cells decreased significantly after gonadotropin stimulation that leads to meiotic resumption [218]. A positive correlation ($r = 0.99$, $P<0.01$) was reported between proportion of pig oocyte undergo meiotic resumption and COCs that lose gap junction [219]. Thus, the oocytes lose an important supplier of nutrients and factors

from cumulus cells playing an important role in cytoplasmic maturation. Furthermore, there is high variation in nuclear morphology of GV stage of pig oocytes obtained from slaughtered ovaries [220]. As a result, some oocytes will start meiosis earlier than other leading to heterogeneity in maturation status [10]. To overcome this problem, many studies have shown that temporary delaying the meiotic resumption during the first part of the IVM period (first 20-22 hrs) can synchronize the progress of maturation and improve the development competence of oocytes [10, 14, 189]. During the inhibition of meiotic resumption, protein synthesis continues in the oocytes [27], accumulating important factors (proteins, mRNA) in the cytoplasm through communications with cumulus cells. The temporary inhibition of meiotic activity can be achieved by using chemicals to elevate the intercellular level of cyclic adenosine monophosphate (cAMP) or to suppress the activity of the maturation-promoting factor (MPF). The following sections describes the different strategies to inhibit meiotic resumptions.

1.5.1 Control of intracellular cAMP level

Studies in different species have been used the strategy of increasing level of cAMP to prevent meiotic resumption and to improve the oocytes competence. Various agents increase the cAMP level by one of the four known mechanisms. First mechanism, the agents (such as dibutyryl cAMP (dbcAMP) and 8-Br-cAMP) has the ability to penetrate into the cumulus cells, and then enter oocytes through gap junctions. The most common chemical used for this group is dbcAMP due to its lipophilic nature that make it highly permeable to intact cells and resistant to hydrolysis by PDE3A[221, 222]. This cAMP analog has been used to maintain meiotic arrest in bovine [223], rat [224], mouse [225] and human [226] oocytes. In pig, many studies have used dbcAMP at a concentration of 1mM [139, 195, 227]. These studies used pre-pubertal porcine oocytes and they were treated dbcAMP for 20-22 hrs, at the beginning of the maturation period [14, 195]. The results showed an increase in meiotic progression and blastocyst formation rate. In addition, this treatment decreases the incidence of polyspermy [10, 11, 14, 73, 189, 227]. The same treatment was also applied on pig oocytes obtained from sows and blastocyst formation did not change between treatment and control [195].

The second way to elevate the cAMP level in oocytes is by the activation of adenylate cyclase (ADYC) in cumulus cells. Many chemicals work as activators for ADYC such as forskolin and cholera toxin [80, 228]. Forskolin has been used widely to inhibit the meiotic resumption in many species [223, 229, 230]. Recently, a study has evaluated the effect of forskolin on bovine

oocytes using different concentration (0.1, 0.05, 0.025 mM) for 6 hrs during IVM. Meiotic inhibition of oocytes with one of these treatment did not generate with the production of more blastocyst compared to control [231]. In pig, forskolin incubated with pre-pubertal porcine oocytes effectively inhibited the meiotic resumption during the IVM [80].

The third mechanism works by suppressing PDE activity, which induces the degradation of cAMP in oocytes. The 3-isobutyl-1-methyxanthine (IBMX) is a PDE inhibitor used to prevent meiotic resumption in bovine [223, 232], rat, and mouse oocytes [233]. In pigs, the addition of IBMX into the maturation medium for the first 20 hrs inhibited meiotic resumption and increased the level of cAMP in oocytes [234]. The fourth group increases cAMP level in oocytes by stimulating cGMP production in cumulus cells. NPPC showed an ability to inhibit meiotic resumption in mouse [100]. Bovine oocytes treated with NPPC (100 nM) had a delay in meiotic resumption during IVM and an increase of blastocyst rate after fertilization of the treated oocytes [235]. In pig oocytes, NPPC was used in the second 22 hrs of IVM, after nuclear maturation of oocytes were synchronized with dbcAMP [236]. The results showed an inhibitory effect of NPPC increased significantly ($P<0.05$, GV rate from 20 to 60%) with an elevation NPPC concentration (10-1000 nM).

1.5.2 Control of intracellular maturation promoting factors (MPF) level

Meiotic resumption is promoted by activation MPF [16]. The phosphorylation of CDK1 is considered as an essential step to activate MPF [90]. Many chemicals have been used to suppress the MPF activity and prevent meiotic resumption such as 6-dimethylaminpurine (6-DMAP, phospherylation inhibitor) [237], butyrolactone-I (BL-I, specific protin-kinase inhibitors) and roscovitine (ROS). BL-I has been used with porcine oocytes [238, 239] and bovine oocytes [240, 241]. BL-I suppresses the activity of MPF by engaging the ATP binding site of CDK1, the catalytic subunit of MPF [242]. In a study on pig oocytes, BL-I was added to maturation media to delay meiotic resumption for the first 24 hrs [243]. Although the results showed a delay in meiotic resumption, no positive effect was found on cleavage and blastocyst rates. Roscovitine also has been used to inhibit meiotic resumption in porcine oocytes via inhibition of cyclin-dependent kinase (p34cdc2/cyclin B kinase) that suppress MPF activity [13]. In bovine, ROS was used in a combination with BL-I at a low dose and was found to delay meiotic resumption effectively without improving embryo development compared to control (untreated) [244].

1.6 Communications between oocyte and cumulus cells

Functions of granulosa and cumulus cells are regulated by factors secreted by the oocyte, such as: growth differentiation factor 9 (GDF9), bone morphogenetic portion 15 (BMP15) or fibroblast growth factors 8 (FGF8) [245]. BMP15 and GDF9 are members of transforming growth factor b (TGFb) superfamily that play a critical role in granulosa and cumulus cells functions [246]. Before LH surge, the BMP15 and GDF9 are produced by the oocyte. They stimulate normal differentiation of cumulus cells [247]. Also, BMP15 promotes metabolic activities in cumulus cells [248] and stimulates the cumulus expansion before ovulation [246]. Oocytes regulate estradiol synthesis by mural and cumulus granulosa cells [249, 250]. Estradiol increases the expression of NPR2 expression [100]. The communication between oocytes and somatic cells have a regulatory loop (Fig. 1.2) that play a significant role in oocytes maturation including meiotic progression and cytoplasmic competence.

1.7 Porcine follicular fluid and defined medium

Porcine follicular fluid (Pff) that is collected from the follicle (4-8 mm) is generally used in IVM media and it is effective at improving the results of embryo development [251, 252]. The porcine follicular fluid contains substances that improve the expansion of cumulus, nuclear maturation and normal fertilization [253]. Many components of Pff have been identified; however, some factors are still not known [254, 255]. For example, Pff contains a high level of superoxide dismutase that plays an essential role in the protection of oocytes against oxidative stress. Superoxide dismutase increases cytoplasmic maturation and oocyte competence [256].
Many studies demonstrated the benefits of using Pff in the maturation media on nuclear and cytoplasmic maturation, normal fertilization, and embryo development [257-259]. The effects of Pff on oocytes competence depend on the size of follicles from which Pff was collected [260]. Pff collected from the large follicle in maturation media had a positive result on oocytes competence compared to using Pff from the small follicle [260]. Interestingly, it was observed that frozen Pff can improve the maturation and normal fertilization of porcine oocytes compared to fresh Pff in maturation media [253]. Although Pff supplement can have the positive effect on oocytes maturation and embryo development, there is a wide variability in the content of Pff obtained from different batches. This variability in Pff causes inconsistent results among studies

[261]. Also, Pff could be a carrier for a pathogen if the follicular fluid is collected from infected pigs [262]. A chemical based medium (defined medium, no Pff or serum) for IVM can help to standardize the media and identify specific metabolic requirements of oocytes with no risk of contamination. In addition, defined medium can contribute to achieve consistent results among laboratories. Many components have been used to replace Pff, including polyvinyl-alcohol (PVA) or poly-vinyl-pyrrolidone (PVP). These substances were used to prepare chemically defined media with TCM-199 [261] or modified NCSU-23 [160].Unfortunately, the results in both studies showed that using these defined medium can allow matured oocyte to develop into lower blastocysts rate compared of using Pff (Defined media vs Pff, 7.7% vs 23.3% [160]; 13% vs 22% [261]).

1.8 Importance of using reproductive technologies in the commercial pig industry

Successful pork production depends on the reproductive performance of the pig. Therefore, reproductive assistance technologies have become an essential tool in animal breeding to improve the productivity performance of swine [141]. *In vitro* embryo production, vitrification, and embryo transfer techniques can play an important role in improving pig productivity [263]. The ability to import and export pig genetic material around the world with minimum health risks would be valuable to the swine industry. Currently, semen (carry half-desired genetics) and live animals are the most common ways of handling genetic materials. Both methods are associated with a high risk of disease transmission [264]. However, embryos carry the full genome and are reportedly very low-risk for disease transmission [264]. Therefore, an *in vitro* embryo production technique could produce large number of viable embryos and it can have a significant role in breeding program with minimum costs when compared with embryos that collect *in vivo*. To make embryos practical for commercial work, cryopreservation using a vitrification technique is used to preserve embryos for short- or long-term use. Thus, reproductive techniques can have a critical role in developing and improving pork production when they are used to improve the genetic progress of pigs.

Successful embryo transfer in combination with other reproductive technologies can significantly contribute to the genetic improvement in pig breeding programs through the ability to introduce new genetic stock as replacement herd animals[265, 266]. It was found that the ultimate impact of using biotechnology, including embryo transfer in pig breeding, can elevate the rate of

progress, efficient use of variation, and reduce genetic lag [265]. Globally operating breeding companies could also benefit from transporting embryos instead of live animals. One of the benefits of using embryos is the lower cost of transportation. Also, they can improve the biosecurity of embryos being transported between countries to minimize the risk of disease transmission [267]. Therefore, using embryos and other reproductive techniques will be more preferable to ensure the health of herd status that represents a critical point in a successful pig industry [268]. The implantation of embryos derived from IVP technique with cloning can produce additional benefits for commercial pig populations [267]. Brascam and haley [263] suggested that using cloning to spread specific genotypes for particular markets can be applied at the level of the nucleus to become a source for cloning in these markets, such as daily growth rate and feed intake capacity. The other advantage of using embryos and vitrification techniques is the ability to preserve embryos of different pig breeds for the long term in order to maintain genetic diversity. Any future genetic improvements in pig breeding depends primarily on having sufficient genetic variation [269]. Genetic diversity can support pig breeds meeting current and future production requirements in different environment conditions. Consequently, this allows for sustained, economically important genetic improvement, as well as the ability to adapt breeding objectives rapidly [270]. Over last 50 years, many pig breeds (109 breeds) became extinct, and 23% of worldwide pig breeds are at risk of extinction [271]. Also, many studies have been reported o the risk of loss of genetic diversity in pig breeds [270, 272, 273]. These facts highlight the significant loss of genetic material globally. Therefore, preserving embryos (full genome) of different pig breeds will allow for maintaining genetic diversity or recreating extinct breeds [274]. As a result, genetic diversity strengthens a population by increasing variation, which allows for improving pig breeds to support pig production.

Although using embryos derived from IVP in the pig industry has many benefits, there are also many limitations for applying this reproductive technique in commercial work. One of the most critical limitations is using surgical procedure for collecting and transferring embryos. Surgical procedures for commercial applications in the pig industry have only been used to a limited degree [275]. All surgical transfers or collections of embryos are performed under general anesthesia, and the animals need time for health care and recovery. Therefore, using embryos for commercial work is very limited in pigs [267]. Additionally, the cost of surgical procedures and

the need for specialists and high levels of training can be other obstacles for applying this procedure.

One of the most key factors in the ability to use embryo transfer technology at a commercial level is the ability to collect and transfer embryos using procedures that can be applied under field conditions. Thus, many groups worked to find non-surgical methods to transfer the embryos, and the results of the attempts were variable. In general, both the farrowing rate and litter size are still rather low [276-279]. However, recently this technique has been developed and has been producing satisfactory results, indicating a promising solution for this problem [280-282]. Also, the efficiency of the IVP procedure in producing viable embryos in pigs is still considered low compared to using IVP in cattle or mouse [283]. A successful IVP procedure is considered an important component for using embryos in pig breeding, in addition to other reproductive techniques [265]. Also, collecting the oocytes for IVM, or embryos from live animals by using endoscopic procedures, has been developing more than regular surgical methods [284-286]. Although this procedure requires few small incisions for instruments, endoscopic procedures still require anesthesia and other surgical precautions [267]. Therefore, endoscopic procedures are less applicable for individual farms, but are considered to be minimally invasive surgical methods of transferring embryos [284].

Overall, the ability to use embryos transfer and other reproductive techniques, such as IVP and vitrification in the pig industry, can support genetic improvement in pig breeds. Also, using different reproductive techniques can enhance the biosecurity of embryos to prevent disease transmission. However, the commercial application of embryo transfer faces big challenges in the pig industry due to the requirement of surgical procedures for both the collection and transference of the embryos. Optimizing reproductive technologies such as non-surgical embryo transfer and IVP can play an important role in supporting pig productivity and quality. The ability to make the embryo transfer (ET) more applicable to use by nonsurgical method safely and under field condition become the interest of many studies that tried to apply ET commercially in pig industry [281]. Recently, non-surgical deep-uterine ET has been developed and showed satisfactory results when using fresh embryos in gilt and sows [287]. Using this technique was able to reach 40% farrowing rate indicating to the promising way that can be used for ET in pig [288]. In addition to ET, preserving pig embryo for the long or short term using vitrification technique represent a critical point in term handling the embryos that were used in

ET. Vitrified pig embryos techniques have developed rapidly using many deceives [289, 290] and showing successfully results in producing live piglets [291]. Having successful tools to preserve the pig embryo and nonsurgical transfer represent a critical point in progress for international trade of pig embryos that can be used for the swine industry. This could lead to convincing pig breeding companies to support research in IVP or embryo transfer to maximize efficiency. These techniques (embryo preservation) can allow us to protect different pig breeds that play an important role in meat production from future extinction, as well as ensuring global food safety.

1.9 Genes transcription regulation in oocytes

The transcription factors and proteins stored in the cytoplasm have an essential role in oocyte maturation and early embryogenesis [292]. During the growing phase of oocytes, transcription is very active, and oocytes accumulate a large amount of mRNA and protein for oocyte maturation and early embryo devlopment [293, 294]. The accumulation of maternal mRNA is required for proper development of the zygote [193]. This transcription and storage of mRNA becomes silent as oocytes transform from the GV to GVBD stage [295]. After transcriptional silencing (regulated by epigenetic mechanisms), oocytes depends on stored transcripts to resume the meiosis and early cleavage division after fertilization [296, 297]. After meiosis resumption, the gene expression in the oocyte is regulated by cytoplasmic polyadenylation. In general, polyadenylation elongates poly (A) tails of some classes of existing mRNA, leading to increased translation and protein level. Deletion of poly (A) tails (deadenylation) is associated with mRNA degradation [298]. Thus, regulation of poly (A) tail length is a key mechanism that regulates the activity of maternal mRNA. Cytoplasmic polyadenylation involves in meiotic maturation of the oocyte in both mouse *and Xenopus* via the translational activation of the mRNAs encoding regulators of meiotic maturation [298]. In bovine, studies on oocytes and embryo indicated that polyadenylation of different mRNA is involved in the meiotic resumption and first embryonic cleavage [299]. It was found that the pattern of polyadenylation can change between the high-quality embryo and low. Also, abnormal polyadenylation can impede the embryo development. During oocyte maturation, the quantity of protein synthesis changes due to either deadenylation or degradation of mRNA [296]. This phenomenon may explain the differences in the global mRNA transcripts in oocytes between GV and MII stage [295]. In human, gene expression (20

genes) was followed over the maturation period of oocytes at different meiotic stages (GV, MI and MII) and the results showed that gene expression varies between the nuclear maturation stages [300]. In bovine, a study evaluated the differences in gene expression between GV vs MII from the adult and pre-pubertal derived oocytes during IVM [301]. It was observed that 695 and 553 genes were differentially expressed in oocytes between GV vs MII stage in the adult and prepubertal groups respectively. Also, same study found that genes were differentially expressed in oocytes between prepubertal vs. adult bovine in GV (333 genes) or MII (549 genes) stages [301]. It is suggested that the gene expression changed over maturation due to the selective deadenylation of maternal mRNA transcripts [42]. The age of a donor for oocytes can also affect gene expression. A study on oocytes derived from pre-pubertal pigs found 59% of differentially expressed genes (587/999 genes) had decreased transcript abundance compared to oocytes derived from cyclic females [302]. While the other 41% of differentially expressed genes (412 out of 999) displayed an increase in oocytes derived from pre-pubertal compared with cyclic females. The gene expression of oocyte can be affected with different conditions including oocyte development stage, microenvironment of oocyte and age of donor.

1.10 Vitrification of pig embryo

Embryo cryopreservation has become an important tool to store porcine genetic material for a long-term period. Many benefits can be gained from this process, including the storage of high–value pigs, protection of genes from disease outbreaks, and reduction in the cost of transport of genetic material. Vitrification is one cryopreservation method that has been applied to oocytes and embryos of various mammalian species [303] including the pig embryo. Vitrification is a process that depends on the solidification of a solution containing a high concentration (4-6 M) of the cryoprotective agent (CPA) inside the cell during rapid cooling. The most common cryoprotectants (CPA) that have been used are ethylene glycol alone or ethylene glycol combined with dimethyl sulfoxide (DMSO) [142]. During the cooling process, cellular fluid converts to a glass-like amorphous state and all cellular motions and biological activities are in a holding pattern [304, 305]. Vitrification minimizes ice crystal formation by passing through critical temperatures very rapidly. However, the utilization of CPA at a high concentration may cause injuries due to cytotoxicity [306]. Successful vitrification depends on two factors: a very high concentration of CPA and a rapid cooling and warming rate (2000-20000 ºC/min). This

technique has been used to successfully vitrify mice embryo without the formation of intracellular ice [305].

Historically, cryopreservation of pig embryos has been challenging due to the high quantity of internal lipid vesicles, which cause chemical or physical damages [307]. Vitrification method overcomes this issue of membrane injuries caused by chilling. As such, it has become the method of choice for the preservation of pig embryos [308]. The first successful piglets born from vitrified embryos were obtained from *in vivo*-derived blastocysts [307, 309]. Over the last 25 years, several vitrification protocols were developed for pig embryos [310, 311]. The differences among these protocols are related to two main points: the vitrification carrier and the cryoprotectants. Vitrification carriers are designed to increase the speed of the cooling/warming rate [312]. It requires a smaller volume of CPA, which increase the cooling rate and improve the heat transfer [313]. Vitrification carriers (cryotop and cryoloop) are opened system that could carry <1 µl of CPA and could allow a direct contact with liquid nitrogen (LN_2) to increase the cooling rate (20000 ºC/min) [313]. Another type of vitrification carrier is a closed system, which is designed to do not have direct contact of the embryo with LN_2. This system was also used to vitrify pig embryos derived from *in vitro* [314] and *in vivo* [311] methods.

Pig embryos derived from IVP are the most likely to survive after vitrification when they are at morula or at a blastocyst stage compare to embryos at a 2-4 cell stage [315]. It is possible that high lipid content at an early embryonic stage decreases as an embryo develops [316]. Therefore, many studies have suggested chemically or physically removing lipid content (delipation) from embryos could improve survival rate after vitrification [317, 318]. One study developed a mechanical delipation protocol by using centrifugation that removed the intracellular lipid from the embryo within the intact zona pellucida [298]. Chemical delipation also could be applied by using phenazine ethosulfate, which reduced the lipid content in the IVP embryo [319].

Culture conditions for an embryo have a role not only in embryo production, but also in cryosurvival for an embryo. Nutrients supplemented in culture media such as serum(SR) [320] or β-mercaptoethanol(β-M) [321] support the success of vitrification of IVP blastocysts by increasing blastocysts survival rate (SR vs Control, 76 vs 67%; β-M vs control, 21.3 vs 18.7%). Another study found that adding l-ascorbic acid into the culture and vitrification media enhances pig blastocyst survival rate by around 10% [321]. Moreover, vitamins in culture media improved the survival rate of the IVP blastocyst by using MEM (Minimum Essential Medium) [322]. In

summary, the vitrification is a cryopreservation method for pig embryos that has been used as a successful tool for a long term storage with minimum cryodamage. Vitrification has an important value in pig industry by using vitrified embryo in improving and protecting pig breeds, embryo transfer, and reducing the cost of importing and exporting valuable breeds without worry of using the live animals. However, the success of vitrification depends on different factors, such as, age of embryo donors, vitrification protocol, and developmental stage of embryos.

CHAPTER 2

2.0 GENERAL OBJECTIVE AND HYPOTHESIS

The general objective of this research was to determine the effect of delay meiotic resumption strategy of pig oocytes during *in vitro* maturation on improving the competence of cytoplasmic maturation to enhance the efficiency of *in vitro* embryo production in pigs to be effectively vitrified using close carrier system.

The general hypothesis is that delaying meiotic resumption during the *in vitro* maturation will improve the fertilization, cleavage and blastocyst rates under an *in vitro* environment and pig embryo that derived from *in vitro* condition will vitrify successfully using close carrier.

2.1 Specific objectives and hypothesis:

2.1.1 Study 1:

EFFECT OF DELAY IN MEIOTIC RESUMPTION USING DIFFERENT SUBSTANCES ON NUCLEAR MATURATION OF PORCINE OOCYTES AND EMBRYO DEVELOPMENT

2.1.1.1 Experiment 1:

Specific Objective: Evaluate the effect of NPPC, cAMP, cGMP, NO, estradiol, progesterone, or testosterone on nuclear maturation of oocytes during IVM.

Specific Hypothesis: Using NPPC, dbcAMP, cGMP, NO, estradiol, progesterone, or testosterone during IVM will inhibit the nuclear maturation progress.

2.1.1.2 Experiment 2:

Specific Objective: Develop a strategy to increase the efficiency of IVM by using one or combination of NPPC, cAMP, NO, estradiol, and progesterone in IVM media to delay meiotic resumption of pig oocytes.

Specific Hypothesis: Delaying meiotic resumption of pig oocytes during IVM of pig oocytes will improves the fertilization rate and blastocyst production.

2.1.2 Study 2:

EFFECT OF DELAYING RESUMPTION OF NUCLEAR MATURATION ON GENE EXPRESSION in PORCINE OOCYTES MATURED *IN VITRO*

Specific Objective: Determine the influence of delaying meiotic resumption protocol for IVM on gene expression of porcine oocytes compared to standard procedures.

Specific Hypothesis: Different treatments dbcAMP, NPPC+estradiol and dbcAMP+NPPC+estradiol delaying meiotic resumption have different effects on different molecular (signaling) pathways and gene expression of matured oocytes.

2.1.3 Study 3:

COMPARISON BETWEEN A CLOSED AND OPEN DEVICES TO VITRIFY *IN VITRO* PRODUCED PIG EMBRYOS

Specific Objective: Evaluate the efficiency of pig morulas to survive after vitrification under two vitrification systems.

Specific Hypothesis: *In vitro* produced embryos would survive the vitrification procedures at an equivalent rate using a closed (French Straws (FS)) or an open device (Open Pull Straw (OPS)).

CHAPTER 3

EFFECT OF DELAYING MEIOTIC RESUMPTION ON NUCLEAR MATURATION OF PORCINE OOCYTES AND EMBRYO DEVELOPMENT

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Author contributions:

Hayder Al-Shanoon: Perform the study, participate in the experimental design, wrote the manuscript

Carl Lessard: Experimental design and critical revision the manuscript

Citation of the paper:

This paper has not been submitted yet

Relationship of this study to the dissertation

In chapter 3, we evaluated the effect of delaying meiotic resumption temporally during IVM on nuclear maturation and embryo development by using different substances (NPPC, cAMP, cGMP, NO, estradiol, progesterone, and testosterone). In chapter 3, we tried to find the best substance or a combination of substances for delaying meiotic resumption, which can improve embryo development and can be used in next study (Chapter 4). We tested two hypotheses that they are: 1- some follicular substances (NPPC, cAMP, cGMP, NO, estradiol, progesterone, and testosterone) can inhibit the nuclear maturation at different levels during IVM; and 2- Delaying the nuclear maturation during IVM of pig oocytes improves the fertilization rate and blastocyst production.

3.1 Abstract

Insufficient cytoplasmic maturation and unsynchronized nuclear maturation during the *in vitro* maturation (IVM) of porcine oocytes reduce their competence to sustain fertilization and embryo development. Delaying nuclear maturation by using natriuretic peptide precursor type C (NPPC), cyclic guanosine monophosphate (cGMP), cyclic adenosine 3, 5 monophosphate (cAMP), nitric oxide (NO), and hormones (testosterone, progesterone and estradiol) could enhance oocyte competence. The goal of this study was to evaluate the effects of these different substances to inhibit nuclear maturation during IVM without affecting the ability of the oocyte to sustain embryo development. Cumulus-oocyte complexes (COCs) were collected from ovaries of slaughtered prepubertal gilts. In the first experiment, different follicular substances (NPPC, cAMP, cGMP, NO, testosterone, progesterone and estradiol) were added to maturation media to evaluate their effects on nuclear maturation of oocytes during IVM. In the second experiment, the usage of these follicular substances was optimized. The effect of these follicular substances on nuclear maturation of oocytes was evaluated at different times (2, 6, 18, and 22 hrs) during IVM. The COCs were incubated for 22 hrs in maturation medium without LH and containing the optimized follicular substance. After this incubation, COCs were transferred into a maturation medium containing FSH and LH for another 22 hrs. Nuclear maturation was evaluated after these two periods of incubation. The results indicated that cAMP (CA, 1mM), the mix of NPPC (1000 nM) and estradiol (100 ng/ml, NE), and the combination of previous treatments (CANE) delayed the meiotic resumption for 22 hrs without effecting the viability of the oocyte. In the last experiment, delaying meiotic resumption with CA, NE, or CANE treatments were applied into the IVP to evaluate the ability of the treated oocyte to sustain embryo development. At day 7 of the culture period, the blastocyst rate did not show any significant differences among the treatments compared to the control (Pff). In conclusion, using NPPC plus estradiol and cAMP and their combination can be considered a promising way to delay the nuclear maturation without having any effects on the final nuclear maturation (MII) of oocytes, and sustain embryo development.

3.2 Introduction

In vitro embryo production, (IVP) is an important technique in improving the genetics of a herd, controlling disease outbreaks, and for long-term preservation of the genetics resource of a breed

[5, 323]. However, IVP in pig still faces many obstacles during *in vitro* maturation (IVM), *in vitro* fertilization and *in vitro* culture. During IVM, oocytes need to complete two important processes: nuclear and cytoplasmic maturation. The completion of these processes ensures that the oocytes will be competent for fertilization and embryo development [27]. One of the most common problems to overcome is insufficient cytoplasmic maturation of oocytes during *in vitro* maturation. This problem is identified as the primary reason for low competence of *in vitro* matured oocytes, leading to abnormal development of the embryo after fertilization [6, 324]. Poor cytoplasmic maturation is a result of insufficient time for the cytoplasm to acquire factors involved in an important process of IVP. This problem is amplified by the variability of the maturation status of COCs derived from different sizes of follicles [24, 325]. Oocytes spontaneously resume meiotic resumption in *in vitro* conditions. It is expected that oocytes collected from ovaries have different nuclear maturation stages and will reach metaphase II at different times, leading to variation in cytoplasmic maturation [324]. Because RNA synthesis and the accumulation of many other important factors are essential for cytoplasmic maturation, timing is a critical issue for oocytes in terms of developmental competence. After oocytes start meiotic resumption , important factors provided by cumulus cells decrease significantly due to the fact that their gap junctions with the oocyte are removed [218]. During oocytes transformation from the GV to GVBD stages, the transcription of mRNA becomes silent [295]. Therefore, the factors that are produced from oocytes participating in oocyte competence and oocyte –cumulus cell communication will be dropped. So, it could be hypothesized that delaying meiotic resumption may give more time for the oocyte to accumulate the important factors required to sustaining embryo development by maintaining gap junction communications between oocyte and cumulus cells.

Meiotic arrest in the oocyte is controlled by the level of cyclic adenosine 3, 5 monophosphate (cAMP) that increases due to signals from follicular cells [73]. Degradation of cAMP is prevented via the inhibition of type 3 phosphodiesterase in the oocyte by cyclic guanosine monophosphate (cGMP) produced in cumulus cells [82, 326]. Also, cGMP has a direct effect on gap junction communication (GJC). It has been observed that cGMP inhibits luteinizing hormone-induced disruption of GJC in rat granulosa cells [117]. Thus, cGMP plays a critical role in meiotic arrest by maintaining GJC between oocytes and cumulus cells, which maintain a high level of cAMP in oocytes. Recently, several studies have indicated an important role of the

natriuretic peptide precursor type C (NPPC) in the meiotic arrest of oocytes of bovine [235], mice [327] and pig [328]. NPPC is produced from mural granulosa cells [97], and natriuretic peptide type 2 (NPR2) receptors are present in cumulus cells [328]. The primary role of NPPC is to stimulate cGMP production in cumulus cells, inhibiting the type 3 phosphodiesterase [69]. Nitric oxide (NO) is also involved in meiotic arrest. In the follicle, endothelial NO synthase (eNOS) of mural cells produces the NO [329], which increases cGMP production from cumulus cells [127]. Meiotic arrest could also be control by steroids. Estradiol and testosterone were found to increase the expression of NPR2 *in vitro* [100]. A high level of testosterone inhibited meiotic resumption in mouse oocytes [138] and support cGMP production [100]. In addition, progesterone was reported to have an inhibitory effect on cAMP phosphodiesterase (PDE) activity, which cause a cAMP degradation in oocytes and lead to an increase of cAMP concentration in oocytes [330]. The goal of this study was to evaluate the effect of follicular substances (NPPC, cAMP, cGMP, NO, estradiol, progesterone, and testosterone) on nuclear maturation of oocytes during IVM and establish a strategy to delay the nuclear maturation to improve the ability of the oocyte to sustain embryo development.

3.3 Material and methods

3.3.1 Media preparation:

All chemicals were purchased from the Sigma Chemical Co, Oakville, Canada. Any chemical that was not from Sigma is noted. All media prepared during the study were filtered with a 0.22 μm filter (Argos technology, Elgin, USA).

3.3.2 Oocyte collection and in vitro maturation (IVM):

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within two hours. Ovaries were dissected from adjacent tissues and rinsed three times in saline at room temperature (0.9% NaCl). Cumulus-oocyte complexes (COCs) were aspirated from follicles that were 3-6 mm in diameter. The COCs were evaluated under stereo microscope to select grade 1 and 2 and transferred into TCM-199 (Invitrogen, Burlington, Canada) supplement with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 25 mM)) and antibiotic-antimycotic (1X). After washing three times using this solution, COCs were

transferred into maturation media (TCM-199 (Invitrogen), 0.1% polyvinyl alcohol , 3.05 mM D-glucose , 0.91 mM sodium pyruvate , antibiotic-antimycotic 1X (Invitrogen), 10 ng/ml EGF, 0.57 mM L-cysteine , 0.01 U/ml Folltropin® (Bioniche, Oakville, Canada)) supplemented with one of the tested substances (treatments). A maximum of 50 COCs per well were used for *in vitro* maturation. Selected oocytes were incubated for 40-44 hrs under 5% CO_2, 5% O_2 and 90% N_2 in a humidified environment at 38.5°C.

3.3.3 In Vitro Fertilization (IVF) and Embryo Culture (IVC):

By the end of the maturation period, oocytes were denuded with 0.1% hyaluronidase in medium-199. Denuded oocytes were washed three times in a modified tris-buffered medium (mTBM) [22]. This media was supplemented with 1 mM caffeine and 0.4% bovine serum albumin (BSA; Minitube,Verona, USA). Washed oocytes were transferred into mTBM that was used as a fertilization medium at a concentration of 50 oocytes/500 µl . For semen preparation, semen straws were thawed at 50°C for 10 seconds and centrifuged with mTBM media twice at 1000 g for 7 min twice. The concentration of sperm added to the oocytes was 0.1×10^6 sperm/ml. The incubation with sperm continued for 6 hours at 38°C in an environment of 5% CO_2, 5% O_2 and 90% N_2. After 6 hrs of incubation, the presumptive zygotes were washed and cultured in NCSU-23 [23] supplemented with 0.4% BSA for 48 hrs. The culture media was modified by replacing glucose with sodium pyruvate (0.33 mM) and sodium lactate (4.5 mM). Subsequently, embryos were transferred into a non-modified culture media (NCSU- 23, with glucose) for another 7 days. The zygotes were incubated in 5% CO_2, 5% O_2 and 90% N_2 in a humidified environment at 38°C.

3.3.4 Assessment of oocyte nuclear maturation:

At the end of the maturation period, COCs were mechanically denuded from cumulus cells by repeated pipetting. They were fixed in 4% paraformaldehyde. Theses oocytes were incubated for 30 min in 0.5% Triton-X 100 in DPBS at room temperature for membrane permeabilization. After the incubation, the cells were washed three times in DPBS+0.1% PVA buffer. Then, they were transferred into 0.05% Tween-20 in DPBS for 30 min at room temperature for re-membrane permeabilization. After this incubation, oocytes were washed three times in DPBS + 0.1% PVA. Then, oocytes were stained using an anti-lamin A/C and 4′,6-diamidino-2-

phenylindole [DAPI] technique [331]. After staining, the oocytes (N for each group ≥ 75 oocytes) were examined with an epifluorescence microscope and classified based on the nuclear status: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) (Fig. 3.1-A).

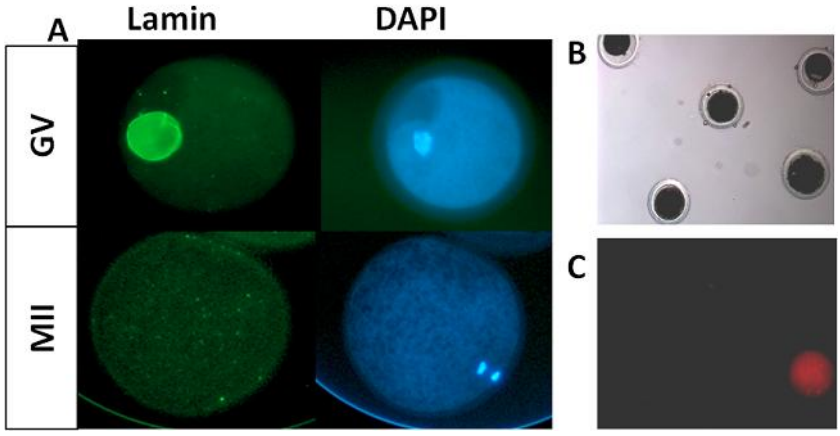


Fig.3.1 Different nuclear maturation stages (GV and MII) in pig oocyte and the viability of oocyte. A) DAPI and LAMIN staining of oocytes: Germinal vesicle (GV) and metaphase II (MII). B and C) Propidium iodide (PI) viability assessment of oocytes by staining the nucleus of oocyte in red (C)(Magnification 400X).

3.3.5 Evaluation of oocyte viability

The viability assessment of oocytes was done based on oolema integrity by using a propidium iodide (PI, Invitrogen) stain. The denuded oocytes were washed in washing buffer (BPS X1+PVA 1%) three times. The staining solution was prepared by adding 12 µM of PI in washing buffer. After the incubation, oocytes were transferred to a staining solution and incubated for 7 min at 37^{o}C in a dark area. The oocytes were then washed three times in the washing buffer. Stained oocytes were observed by fluorescence microscopy (400X). The dead oocytes stained in red florescence due to disruption of cellular membrane (Fig. 3.1-C), while viable cells were not stained (Fig.3.1-B). The proportions of viable oocytes cultured in different treatments are presented as mean ±SEM (Fig. 3.3).

3.3.6 *Evaluation of cumulus expansion:*

Cumulus expansion was assessed at the end of IVM using the subjective scoring method based on degree of expansion described by Tao *et. al.* [118] with modifications. Cumulus expansion was classified into four categories: no expansion (0, the cumulus cells (CC) are compact around oocytes), minimum response (1, slight expansion of cumulus cells), medium response (2, all outlayers of CC are expanded except for the CC layers around the oocyte), complete expansion (3, all cumulus layers are expanded). The cumulus expansion was scored as one value according to the majority (≥70%), since most cumulus-enclosed oocytes in a well had the same level of expansion [118, 332-334].

3.3.7 Experimental Design

3.3.7.1 Experiment 1: Effect of different follicular substances on nuclear maturation of porcine oocytes

The first experiment was undertaken to evaluate the dose response of various substances on oocyte nuclear maturation and cumulus expansion (assessed at the end of IVM). Oocytes were submitted to:

1- NPPC at a concentration of 100, 250, 500, or 1000 nM.

2- NO donor (S-nitroso-N-acetyl-DL-penicillamine) at a concentration of 0.5, 0.75, 1, 5, or 10 mM.

3- Dibutyryladenosine 3': 5' cyclic monophosphate (dbcAMP) at a concentration of 0.5, 1, or 2 mM.

4- cGMP at a concentration of 2, 5, or 10 µM.

5- Testosterone at concentrations of 500, 750, or 1000 ng/ml

6- Estradiol at a concentration of 100 ng/ml

7- progesterone at a concentration of 100 ng/ml

NPPC, dbcAMP, NO, and cGMP were prepared by dissolving in TCM 199 and storing at -20°C until used. The steroid hormones (testosterone, estradiol and progesterone) were dissolved in

100% ethanol, and then stored at 4°C. The doses of progesterone and estradiol (100 ng/ml) were selected based on the previous study done in our lab to evaluate their effects on nuclear maturation [335]. All the substances were added to the maturation media from the beginning of the IVM period to the end. At the end of IVM, cumulus expansion and viability rate were evaluated, and oocytes were stained to assess the nuclear maturation stages.

3.3.7.2 Experiment 2: Evaluate the effect of delaying meiotic resumption at different times of IVM on nuclear maturation, and embryo development

The aim of experiment 2.1 was to evaluate the ability of selected folliclular substances at an optimal concentration to delay meiotic resumption at certain times (2, 6, 18, 22 and 44 hrs) during IVM. NPPC (1000 nM), dbcAMP (1 mM), NO (1 mM), estradiol and progesterone (100 ng/ml) were used alone or as a combination during the first 22 hrs of IVM in a maturation media (without LH). A portion of oocytes after a predetermined time of incubation (2, 6, 18 and 22 hrs) were fixed and stained to assess the nuclear maturation stage. After the incubation with the follicular substance, the remaining oocytes were washed and transferred into a regular maturation media (with LH). *In vitro* maturation of oocytes continued until the total time reached 44 hrs. At the end of the maturation period, a group of oocytes (n=25/ replicate) was stained to evaluate the nuclear maturation stage.

The second experiment (2.2) was performed to evaluate the effect of the selected substances on *in vitro* fertilization and embryo development. Three treatments groups were designed based on the functions during folliculogenesis: dbcAMP, NPPC+estradiol, or NPPC+estradiol +dbcAMP. These substances were added to maturation media (without LH) for the first 22 hrs of IVM. Then, oocytes were transferred to maturation media (with LH) for another 22 hrs. The control group used was 10% Pff in a maturation media for 44 hrs. By the end of the maturation period, oocytes were used for IVF and IVC to evaluate the effect of delaying meiotic resumption on the cleavage and blastocyst rate. The quality of embryos (blastocyst) were evaluated based on the Bo and Mapletoft [336]. Briefly, grade 1 (G1) denotes good quality embryos that have symmetrical and spherical mass with individual blastomeres, viable embryonic mass, and less than 15% of irregular cellular materials. The embryo has a central cavity, big size, and thin zona pellucida. In grade 2, the embryos have moderate irregularities in the overall shape of the embryonic mass and

50% of the embryonic mass should be in intact. While grade 3 embryos represent the poor quality that has main irregularities in the shape of the embryonic mass or in size, color, and density of individual cells. There is a small cavity and at least 25% of embryo mass must be intact. The data of embryo in grade 3 were not considered in this study due to their low quality.

3.3.8 Statistical Analysis

Statistical analysis was done using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Each experiment was replicated 3-5 times. All data are presented as mean ± SEM. In experiment 1, the differences between treatments in each nuclear maturation stage and viability rate were analyzed by one-way ANOVA. For experiment 2, the proportion of oocytes in each maturation stage, and embryonic cleavage (Day 2) and blastocyst rates (Day 7) were analyzed by one-way ANOVA. All the analysis were followed by Fisher's least significant difference (LSD) test and a *P*-value ≤ 0.05 was used to consider different between treatments.

3.4 Results

3.4.1 Experiment 1: The inhibitory effect of different substances on nuclear maturation of porcine oocytes

3.4.1.1 Inhibition of nuclear maturation

At a concentration of 1000 nM, NPPC significantly increased ($P<0.001$) the rate of oocytes in GV stage compared to other concentrations and control (Pff) (Fig 3.2-A). Also, treatment with dbcAMP in a concentration of 1 or 2 mM resulted in a higher proportion rate ($P < 0.001$) of oocytes in the GV stage compared to other treatment (0.5 mM) or control. In addition, the 1 and 2 mM of dbcAMP inhibited significantly the rate of oocytes to reach MII stage compared to control or 0.5 mM treatment. With NO treatment, the concentration of 1mM significantly ($P < 0.001$) inhibited the nuclear resumption by increasing oocyte in GV stage compared with other concentrations and control. It was also observed that the different NO concentrations result in significant reduction in oocytes in MII stage ($P < 0.001$) compared to control. Although all of the cGMP treatments (Fig. 3.2-D) showed a higher rate of the oocytes in GV stage ($P = 0.03$) compared to control, the rate of oocytes in the GV stage was less than 20% for every concentration. With steroid treatments (testosterone, progesterone, and estradiol), there was no significant different between treatments and control in all concentrations (Fig. 3.2- E, F).

However, it was observed that the level of oocytes in MII dropped significantly in all hormones treatments compared to control.

3.4.1.2 Viability rate and cumulus expansion

There were no differences in the viability rate of oocytes treated with cAMP, cGMP, testosterone, estradiol and progesterone compared to control or among the tested concentrations within the same treatment. With NO treatments, high concentrations (5, 10 mM) decreased the viability rate significantly ($P < 0.001$) while the viability rate with low NO concentration (0.5-1 mM, Fig.3.3-B) did not differed ($P = 0.05$) compared to control. In NPPC treatments, the Pff group and 0.5 nM NPPC had a higher viability rate ($P < 0.001$, Fig 3.3-A) of oocytes compared to other NPPC concentrations. The cumulus expansion degree of COCs was numerically decreased with all treatments compared to control (Pff). The degree of cumulus expansion in high concentrations of NPPC (1000 nM), cAMP (1, 2 mM) was lower compared with other treatment concentrations and control. The NO treatment recorded the lowest cumulus expansion degree compared to control.

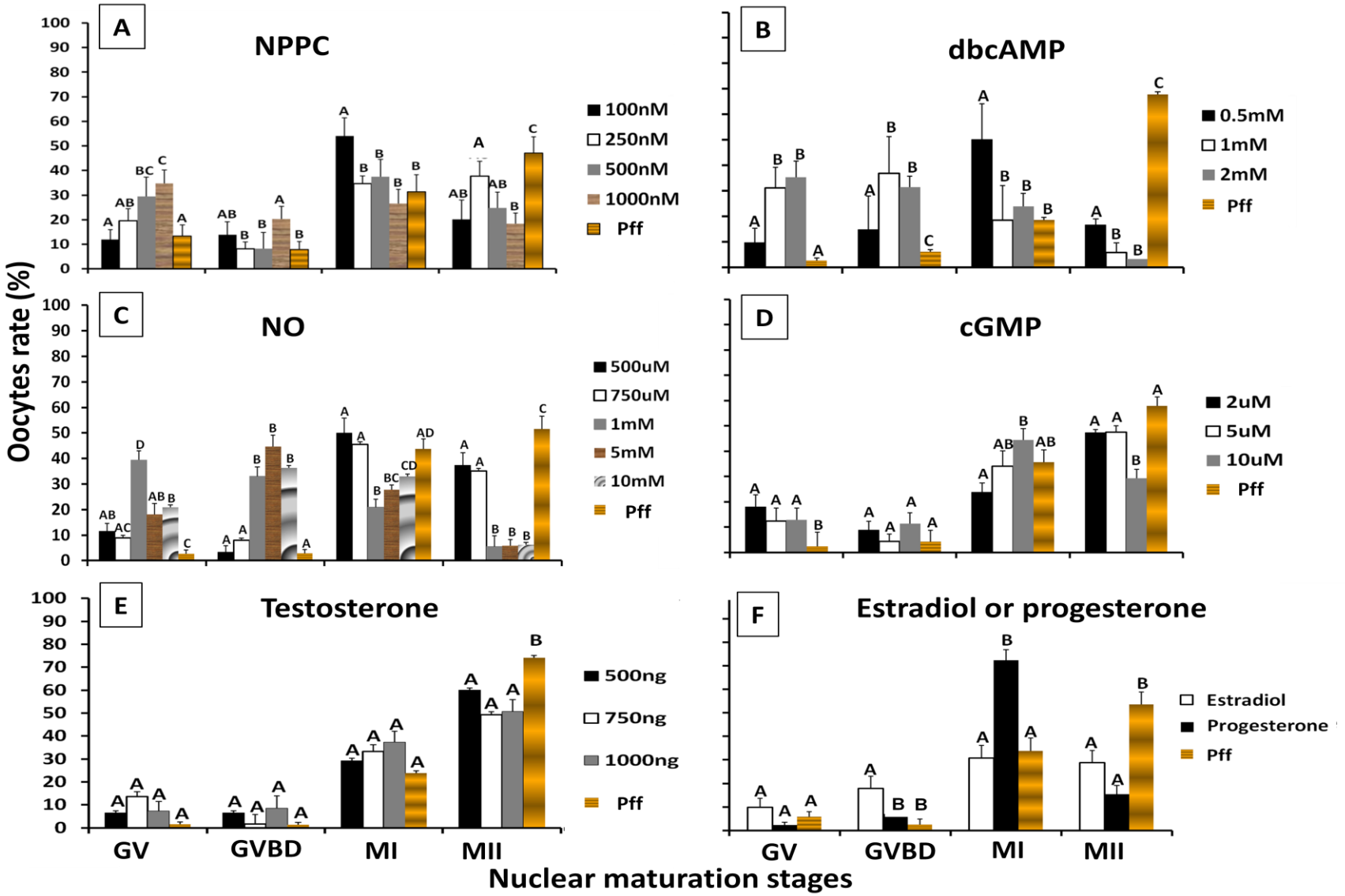

A
NPPC
100nM
250nM
500nM
1000nM
Pff
B
dbcAMP
0.5mM
1mM
2mM
Pff
C
NO
500uM
750uM
1mM
5mM
10mM
Pff
D
cGMP
2uM
5uM
10uM
Pff
E
Testosterone
500ng
750ng
1000ng
Pff
F
Estradiol or progesterone
Estradiol
Progesterone
Pff
Oocytes rate (%)
GV
GVBD
MI
MII
Nuclear maturation stages

Fig. 3.2 Effect of NPPC, NO, cAMP, cGMP, testosterone, estradiol, and progesterone on nuclear maturation rate of oocytes (means ± SEM) after 44 hrs of maturation. Immature oocytes (germinal vesicle and germinal vesicle breakdown) were compared with mature oocytes (metaphase I and II) to highlight the dose effect of these substances on the nuclear maturation progress. Value with different superscripts for same stage of nuclear maturation is significantly different at $P \leq 0.05$. NPPC= Natriuretic peptide type C, NO= Nitric oxide, cAMP= Cyclic adenosine monophosphate, cGMP= Cyclic guanosinemonophosphate. Pff = Porcine follicular fluid.

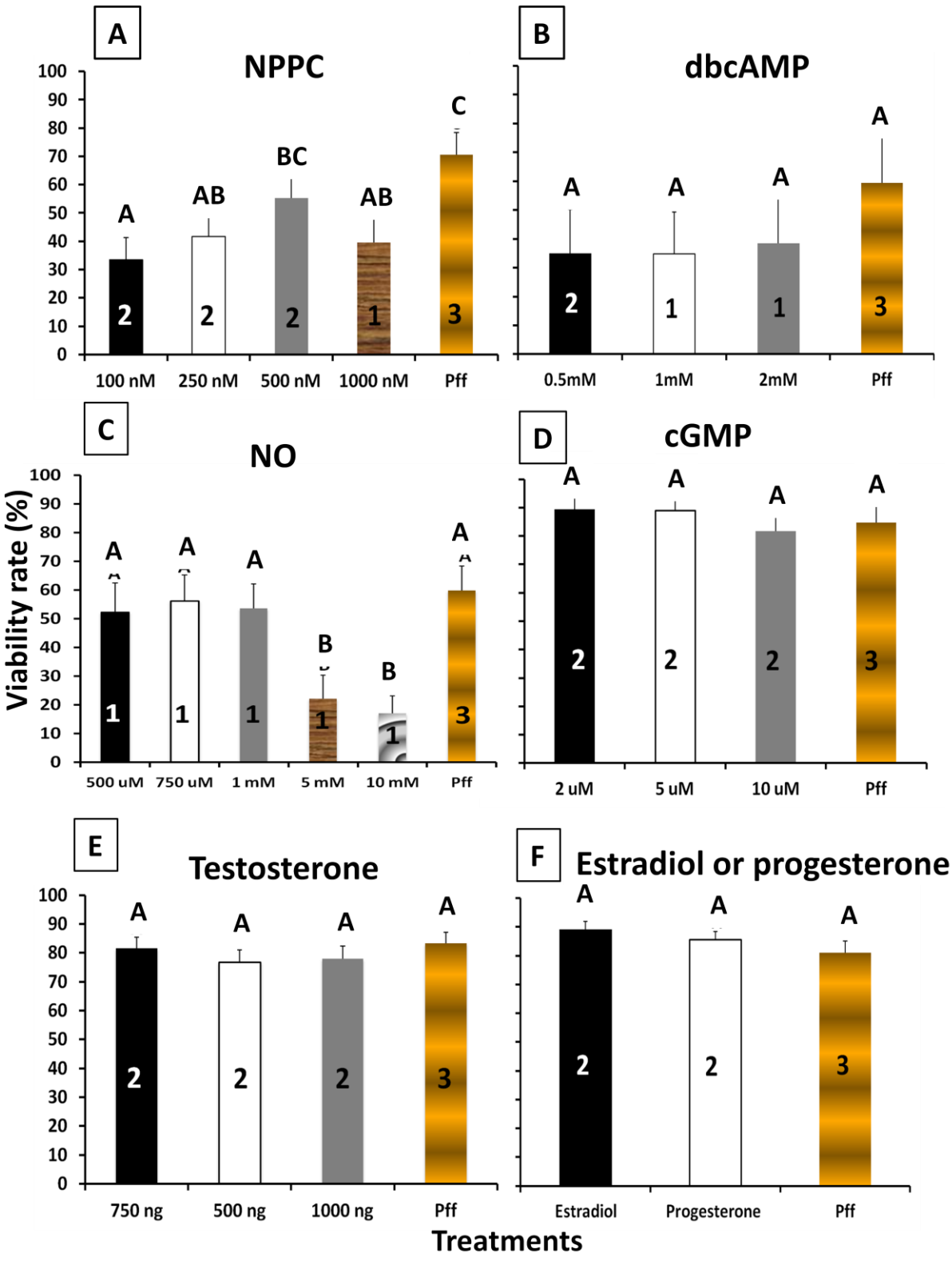


Fig. 3.3 Effect of different doses of NPPC, NO, cAMP, cGMP, testosterone, estradiol, progesterone on the viability rate (means ± SEM) and cumulus expansion degree (white number inside the box) of

matured oocytes. Values with different superscripts within a substance are significantly different at $P \leq 0.05$. Cumulus expansion degree was scored as one value according to the majority since most cumulus-enclosed oocytes in a well had the same level of expansion

3.4.2 Experiment 2: Evaluate effect of delaying meiotic resumption on nuclear maturation at different times during IVM of porcine oocytes

3.4.2.1 Delaying meiotic resumption

The results of Pff and maturation media group (no treatments) showed that the proportion of oocytes in the GV stage dropped significantly ($P = 0.01$) at 18 and 22 hrs of IVM respectively. The rate of oocytes in MII stage for Pff and maturation media at 44 hrs was 64.1% and 44.2 %, respectively. In addition, all substances that showed an inhibitory effect on meiotic resumption were used as a combination in maturation media (NPPC,1000 nM; cAMP,1 mM; NO,1 mM; estradiol and progesterone; 100 ng/ml) to evaluate the maximum inhibition effect on meiotic resumption. By the end of IVM (44 hrs), incubation of COCs with this combination for 6, 18, and 22 hrs (without LH) resulted in 54.1%, 75.9% and 72.9% matured oocytes at GV stage, respectively. In comparison, when NO was removed from this combination, the number of oocytes at the GV stage dropped to less than 15% (appendix, 8.2). These results indicated that NO prevented the oocytes from reversing to meiotic resumption.

For this reason, the combination (without NO) were evaluated at 2, 6, 18 or 22 hrs. The results (Fig.3.4-B) showed that over 80% of the oocytes were arrested at GV stage in the first 22 hrs of IVM. The proportion of oocytes in MII by the end of IVM using the mix without NO was approximately 50% for the 22 hrs treatment group compared 60% with the control (Pff). Then, the substances were evaluated individually over IVM. The results of using estradiol, progesterone, NPPC and NPPC+ progesterone showed a decrease in oocytes in the GV stage over the first 22 hrs of IVM (Fig. 3.4). However, a combination of NPPC+estradiol arrested oocytes at the GV stage (>80%) for 22 hrs of IVM (Fig. 3.4-D). After removing these substances, the MII rate for this treatment at 44 hrs was 62.61% and it was not significantly different from Pff group (Fig. 3.5-A).

3.4.2.2 Effect of delaying meiotic resumption on embryo development

Based on the results of the previous study (section 3.4.2.1), we found that NPPC + E, cAMP and the combination of NPPC, E, and cAMP prevented the meiotic resumption over 22 hrs effectively with more than 80% of oocytes in the GV stage. In addition, these treatments did not affect the final nuclear maturation rate of treated oocytes (MII) compared to control (Pff). Therefore, these treatments (NPPC +E, cAMP and combination of NPPC +E+cAMP) were selected for IVF-IVC procedure. This experiment (2.2) evaluated the effect of delaying meiotic resumption on embryo development. The Pff treatment was used as a control for the IVM procedure. The results of cleavage and blastocyst rates (Table 3.1) did not show any differences between treatments groups and the control.

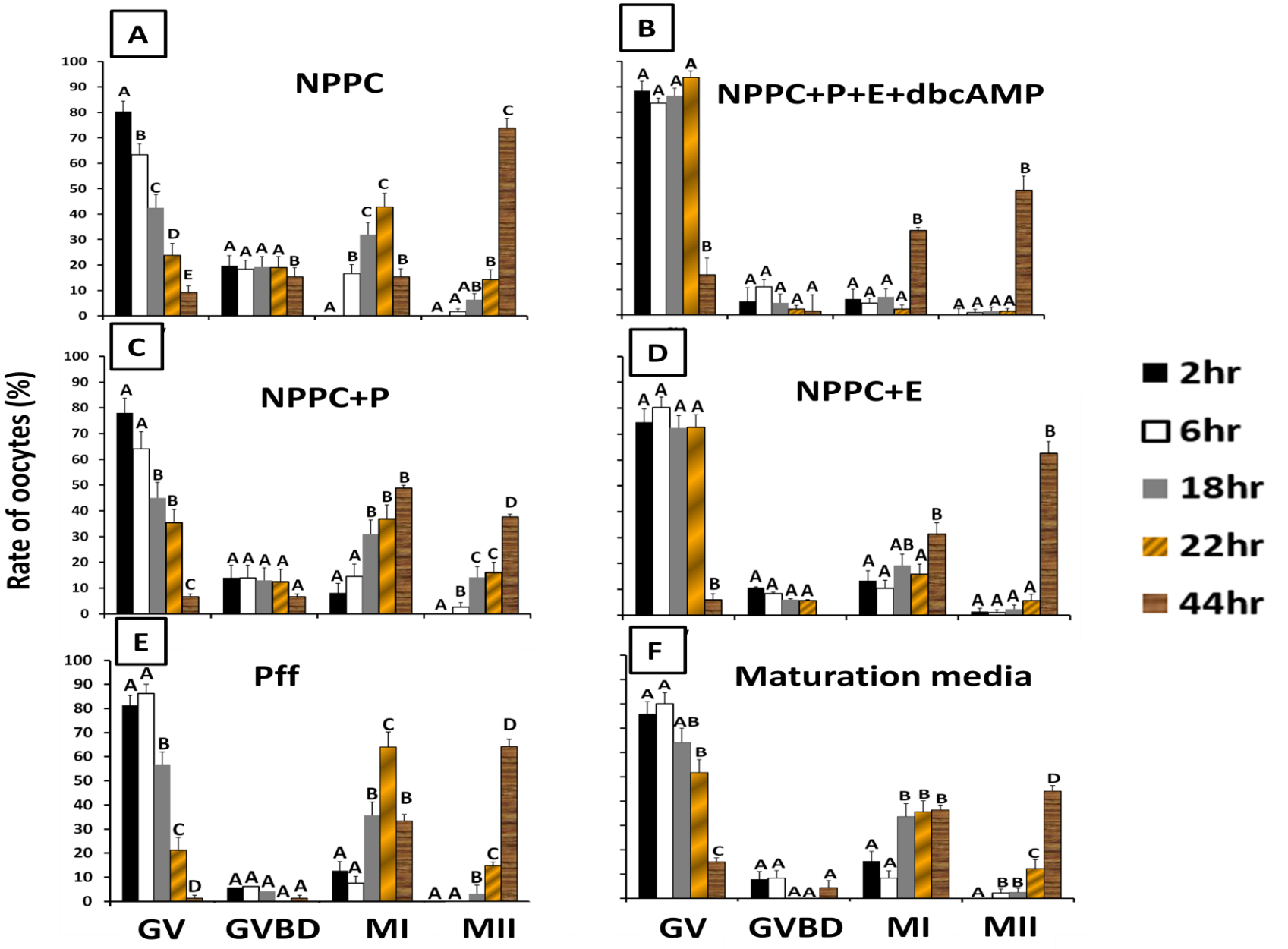
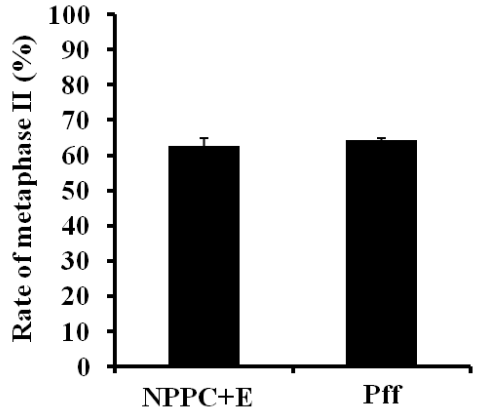

A
NPPC
B
NPPC+P+E+dbcAMP
C
NPPC+P
D
NPPC+E
E
Pff
F
Maturation media
Rate of oocytes (%)
GV
GVBD
MI
MII
2hr
6hr
18hr
22hr
44hr

Fig. 3.4 Effect of different substances alone or in combination on nuclear maturation of porcine oocytes. The mix was added to maturation media (without LH) at the beginning of IVM. After pre-determined times (2, 6, 18, 22 hrs) the oocytes were fixed. After a 22 hr of maturation, a group of oocytes was transferred to regular maturation media (with LH) to complete the maturation process. By 44 hrs, the oocytes were fixed for staining to evaluate the proportion of MII. NPPC= Natriuretic peptide type C, NO= Nitric oxide, cAMP= Cyclic adenosine monophosphate, Pff= Porcine follicular fluid, GV = Germinal vesicle, MII= Metaphase II. Values with different superscripts for the same stage of nuclear maturation are significantly different at $P \leq 0.05$.

A



B

Time of incubation	Number of oocytes	Viability rate
2hr	146	91.6±3.61
6hr	109	85.7±5.57
18hr	132	85.7±5.45
22hr	108	88.3±4.85
44hr	121	91.6±3.78

Fig. 3.5 Effect of delaying meiotic resumption in pig oocytes using NPPC+ estradiol (E) treatment for 22 hrs on final nuclear maturation stage (MII) and oocytes viability**.** A) The metaphase II rate of matured oocytes in NPPC+ estradiol treatments compare to Pff group by the end of IVM (44 hrs). B) The viability rate of matured oocytes in NPPC+ E treatments at different times during IVM. There are no statistical differences among the treatments in different parameters.

Treatment	Total cells	cleavage rate (%) at Day 2	Blastocysts formation rate (based on total cells)	Blastocysts formation rate (based on cleaved cells Day2)	Grade 1&2 from total blastocyst number (%)
cAMP	84	41.7±5.38	7.2±2.84	17.2±6.37	4/6 (66.6)
NPPC+E	80	40.0±5.47	8.7±3.15	21.9±7.31	5/7 (71.4)
NPPC+E+ cAMP	100	35.0±4.77	6.0±2.37	17.2±6.37	4/6 (66.6)
Pff	107	53.3±4.82	9.4±2.82	17.6±5.04	6/10 (60)

Table 3.1: Ability to sustain embryo development on oocytes treated with different meiotic inhibitory substances, NPPC= Natriuretic peptide type C, E= Estradiol, cAMP= Cyclic adenosine monophosphate, CANE =Natriuretic peptide type C+ E+ cAMP, Pff= Porcine follicular fluid. There are no statistical differences among the treatments in different parameters.

3.5 Discussion

Recent studies have highlighted the role of NPPC/NPR2 or nitric oxide-cGMP pathways in meiotic arrest [69, 97, 117, 328, 337]. Many elements of these pathways have been identified such as cGMP, cAMP, estradiol, and testosterone [69, 100]. In this study, we evaluated the inhibitory effect of using NPPC, cAMP, cGMP, NO, estradiol, testosterone, and progesterone on nuclear maturation. The results of experiment 1 (Fig. 3.2) showed that 1000 nM NPPC increased the inhibitory effect on oocytes compared to other concentrations. Another study [328] found that 1000 nM of NPPC inhibited meiotic resumption in porcine oocytes compared to untreated. The inhibitory effect of NPPC on meiotic resumption was also confirmed in the mouse [97], bovine [338], and goat [339].

The cGMP messenger is the key to regulate the level of cAMP, inhibiting phosphodiesterase 3A enzyme in oocytes [82]. The cGMP is known to diffuse through the plasma membrane to increase its level in cells [132]. cGMP analogs have been used to inhibit meiotic resumption in mouse [98], and rat [340]. In this study, any tested concentrations of cGMP did not prevent meiotic resumption (Fig. 3.2-D). A recent study in porcine oocytes found that the ability to inhibit

meiotic resumption by cGMP was lost when FSH was present in the maturation media during the IVM process [131]. For this study, we can hypothesize that the level of cGMP used in this study could not prevent meiotic resumption due to the essential presence of FSH. Interestingly, a recent study found that using a high level of cGMP (5 mM) can inhibit meiotic resumption in presence of FSH, while lower concentrations (0.05 and 0.5 mM) could not [132]. For our study, we avoided using high concentration due to a potential effect on the viability of oocytes.

As a high level of cAMP in the oocyte is the key for a meiotic arrest, many studies have used the cAMP analog (dbcAMP) *in vitro* to prevent meiotic resumption [10, 89, 227]. In this study, dbcAMP inhibited meiotic resumption at a minimum concentration of 1 mM (Fig. 3.2-B). This result concurred with several studies that used dbcAMP as a treatment to delay meiotic resumption [10, 14, 227, 341, 342]. This process has been found to improve cytoplasmic maturation in oocytes and embryo development [324]. In experiment 1, cumulus expansion of oocytes at higher concentrations (1 and 2 mM) of cAMP (Fig. 3.3- C) was lower than at a low concentration treatment (0.5 mM) and Pff. These results are in contradictions with the results of Vanderhyden [332] who found that treating porcine COCs with increasing dbcAMP concentration (0.05 – 2 mM) improved cumulus expansion. In Vanderhyden's study, they used mural granulosa cells in addition to COCs during IVM. It was found culturing mural granulosa cells in maturation media with gonadotropin (LH or FSH) increase progesterone production from these cells over the time [343]. It has been reported that an increase level of progesterone in maturation media could stimulates meiotic resumption in pig COCs [344]. Therefore, it is possible that cumulus expansion in Vanderhyden's study happened due to an elevation in progesterone leading to meiotic resumption.

Also, we found that using testosterone, progesterone or estradiol did not prevent meiotic resumption at any concentrations when used alone. However, estradiol has been reported that plays a role in maintaining oocytes in meiotic arrest [345]. An *in vivo* study in pigs found that estradiol concentration after LH surge rapidly declines from 20 to 1 pg/ml [346], which could suggests that estradiol may be important during meiotic arrest. Estradiol can up-regulate gap junction communications [347], which maintains transport of meiotic inhibitory factors (such as cAMP and cGMP) from cumulus cells to oocytes. In addition, many reports have been observed the positive role of estradiol or progesterone on cytoplasmic maturation and embryo development [348-351]. Several studies confirmed that using appropriate additives to IVM media can increase

the development competence [352-354] indicating that would be possible to improve the development competence during meiotic arrest. Therefore, estradiol and progesterone was also selected to use in experiment 2 and evaluated its effect as combination with other substances (dbcAMP, NPPC and NO) that show ability to delay nuclear maturation.

In the second experiment, we tried to determine the best combination of substances selected from experiment 1 to optimize the ability to delay the meiotic resumption. The result of our study from experiment 2 found the combination of NPPC + estradiol was effective at inhibiting meiotic resumption for 22 hrs without affecting the final MII rate (Fig. 3.4-D). The inhibitory effect of NPPC on porcine oocytes has already been reported [328]. A study on pig oocytes demonstrated that a minimum of 10 µM of NPPC in a FSH-induced maturation medium is enough to inhibit meiotic resumption [132]. In our study, NPPC alone was not efficient to fully inhibit meiotic resumption and present of estradiol supported NPPC ability to prevent meiotic resumption. Estradiol seems to increase the mRNA expression of *NPR2* (NPPC recpters) in cumulus cells of mouse, goat and pig COCs [100, 339, 355]. A study using porcine oocytes found that *NPR2* expression increased significantly when estradiol was used in presence of FSH in maturation media compared to use of estradiol or FSH alone [355]. Thus, a maximum expression of NPR2 could allow NPPC to work efficiently to inhibit meiotic resumption, which could explain the optimal results of NPPC and estradiol obtained in our study. It was also observed using progesterone as a combination with NPPC and E (NPPC+P; Fig. 3.4-C or NPPC+P+E; data not shown) did not prevent meiotic resumption. The possible explanation for this case is progesterone down regulate the gap junctional communication [356]. This drop in communication result from the reduction of connexion43 mRNA expression in cumulus cells, as caused by progesterone receptors[357]. This condition can lead to close of gap junctional communication that induces GVBD on porcine oocytes used in our study. In consequence, many components that produce in cumulus and have role in inhibiting meiotic resumption such as cGMP will not able to transport to oocytes to prevent meiotic resumption.

As our goal was to find a treatment that delayed the meiotic resumption for maximum tested time (22 hrs), three treatments (CA, NE, and CANE) were selected for use in the two-step strategy. These treatments did not affect the starting meiotic resumption after they were removed from the media and the final nuclear maturation (MII) was equivalent to control. Therefore, these treatments were used in the IVF-IVC procedure. We observed that 22 hrs of inhibition with NE,

CA, or CANE can result in embryo development rates equivalent to results with Pff. This optimized time is longer than what has been reported in mouse [358] and bovine [359, 360]. It could suggest that the metabolism activities of the pig oocytes are lower than other species. Interestingly, no differences among treatments groups (Table 3.1) in cleavage or blastocyst rate were observed. Thus, our different treatment group can offer a basis to develop a defined medium without animal products to reduce the variability induced by Pff or enhance biosecurity in the production of germplasm.

In summary, our study indicated that NE, CA, or CANE can effectively inhibit meiotic resumption without affecting the viability rate or final maturation rate (MII), which provides a promising method to delay meiotic resumption in a two-step strategy and improve the quality of the *in vitro* produced embryos to level equivalent of using Pff treatment.

CHAPTER 4

EFFECT OF DELAYING RESUMPTION OF NUCLEAR MATURATION ON GENE EXPRESSION IN PORCINE OOCYTES MATURED *IN VITRO*

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Stephen Tosi: Training and participate in microarray work

Michael K Dyck: Experimental design of the microarray and critical revision for manuscript

Carl Lessard: Experimental design and critical revision for manuscript

Citation of the paper:

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A copyright statement:

Relationship of this study to the dissertation

In the previous study (Chapter 3), different substances have shown a high ability to delay meiotic resumption successfully, and three treatments were selected: NE, CA, and CANE. These treatments showed an equivalent effect to that of Pff on embryo development. This study was focused on evaluating how these treatments can affect gene profiles of matured oocytes during IVM. The changes in gene expression and pathways could help to understand how the treatments can affect oocyte competence positively to improve embryo development, which can reflect positively on the development of IVP procedures. Microarray technique was used to evaluate the gene profiles.

4.1 Abstract

This study was designed to evaluate the influence of a delayed meiotic resumption on gene expressions and molecular pathways of matured oocytes when compared to standard procedures of *in vitro* maturation using porcine follicular fluid (Pff). Based on previous studies, three treatments to delay meiotic resumption were tested during the first 22 hrs of IVM of pig oocytes: 1) Natriuretic peptide precursor C (NPPC 1000nM) plus estradiol (E, 100 ng/ml; NE); 2) dbcAMP (1mM; CA) in maturation medium and 3) a combination of previous treatments (NPPC+E+dbcAMP; CANE). Obtained from pig prepubertal ovaries, cumulus oocytes complexes (COCs) that have ≥2 layers of cumulus cells and homogenous cytoplasm were selected and transferred to one of the treatment groups for 22 hrs. After that, COCs were re-transferred into a maturation medium containing LH for another 22 hrs. In control group, the COCs were incubated into a maturation medium containing Pff (10%) for 44 hrs. The total RNA of the matured oocytes was extracted, amplified, labeled, and hybridized to the gene expression microarray developed for studies with early stage porcine embryos [361] (treatment vs. Pff) using dye swap method. A log 2-fold differences in gene expression between a treatment and reference control (Pff) was considered statistically different ($P < 0.05$). EASE program was used to identify biological theme of the differentially expressed genes of each group. While DAVID and PANTHER programs were used to most significant biological pathways in each treatment group. For all these programs, a P value ≤ 0.05 was used to determine the significance for the gene list. The number of differentially expressed genes for NE, CA, and CANE compared to the Pff group was 122, 55, and 167 respectively. The results of EASE program showed that CA treatment up-regulated many genes involved in signal transduction (ARHGAP9, RGS5, BRAP, STAT2 and 4), chromosome segregation (TTK and RFC3), mitochondrial activity (TIMM9, MRPS30, MRPL3, and IDH3B) and oocyte competence (MRPL3 and HSP90AA1). Using Panther program, our analysis demonstrated that this treatment up-regulated pathways related to signal transduction (JAK/STAT pathway). The CA treatment down regulated interleukin -18 genes. For the NE group, some of these genes were related to MAPK pathway (MAX, MAPK9, REL) or the ERK pathway. Our EASE analysis revealed that the NE treatment had up-regulated many genes related to nucleus metabolism. Our DAVID analysis revealed that the NE treatment down regulated genes involved in fatty acid (SCD5), lipid (PLA2G7), and tyrosine (MIF) metabolisms or ATP synthesis (ATP5B). The last treatment (CANE) up regulated genes (EASE program) that regulate

metabolism (APOA1, APOD, ACO2, CKMT2, DNAH14, and NDUFB7) and growth (IGF1 and ID4). Meanwhile, CANE treatment suppressed genes with roles in signal transduction pathways (IL6, HBEGF, and TLR7).

In conclusion, this study demonstrated that delaying meiotic resumption of pig oocytes with one of the tested treatment resulted in a unique molecular profile that could lead to a positive embryo development after fertilization.

4.2 Introduction

The efficiency of *in vitro* embryo production (IVP) technique in pigs is low compared to the efficiency of IVP of embryos in cattle [362]. The main obstacle for IVP efficiency is poor cytoplasmic maturation during *in vitro* maturation (IVM) process, which leads to the production of incompetent oocytes for fertilization and embryo development [5]. Early resumption of nuclear maturation and removal of the gap junctions do not allow for an appropriate amount of time to acquire a proper level of factors to improve the competence of the oocytes [324]. Recently, strategies to delay nuclear maturation during *in vitro* maturation (IVM) demonstrated an improvement of oocyte competence for cattle [363], goat [364] and pig [89]. Delaying resumption of meiosis increases the period of communication between oocyte and cumulus cells, thereby increasing the exchange of many factors supporting cytoplasmic maturation [324]. Many substances have been used to delay the resumption of meiosis, including dbcAMP (dibutyryl-cAMP) and the natriuretic peptide precursor type C (NPPC) [235, 236, 359]. The dbcAMP was used during IVM to improve the competence of oocytes and embryo development in pigs and cattle [89, 195, 365]. Our research group have recently demonstrated that a combination of NPPC plus E (first 22 hrs of IVM period) efficiently delayed meiotic resumption and resulted in embryo development rates equivalent to using porcine follicular fluids in IVM (Chapter 3). This suggests that a defined medium (laboratory based) can be developed by using different substances to delay meiotic resumption to produce pig embryos. Although most of the results of delaying the meiotic resumption strategy on oocytes during IVM have been promising [10, 11, 359], no studies have yet reported the effect of a delayed meiotic resumption procedure on the gene expressions and related molecular pathways in oocytes.

It was reported that molecular conditions of oocytes or cumulus cells could be affected by adding substances to the maturation media [366-368]. For instance, an increase of Pff supplementation in

maturation media (from 1 to 10%) resulted in the up-regulation of factors involved in the maturation of oocytes such as: matrix molecules, steroidogenesis, and cell cycle regulators [369]. These factors resulted to an improvement in the embryo *in vitro* conditions. Many genes play a key role in oocyte competence and understanding the molecular changes associated with the maturation of oocytes under the influence of inhibitory factors could allow for the identification of new markers associated with oocyte competencies [370-372]. The aim of this study was to determine the influence of an optimized protocol for IVM on gene expression in porcine oocytes compared to standard procedures.

4.3 Material and methods

4.3.1 Chemicals and Media

All the chemicals and media were purchased from the Sigma-Aldrich Company (Oakville, Canada). Chemicals that were not purchased from Sigma were noted.

4.3.2 Oocytes Collection

Ovaries were collected from slaughtered prepubertal gilts at a local abattoir and transported to the laboratory in a thermos box containing gel-packs warmed to 37°C within two hours. The ovaries were then cleaned of adjacent tissues and rinsed in saline (0.9% NaCl) at room temperature to remove blood contamination. The cumulus-oocyte complexes (COCs) were aspirated from the follicles (3-6 mm in diameter). The COCs with ≥ 2 layers of cumulus cells and homogenous ooplasm were selected and washed three times with TCM-199 (Invitrogen, Burlington, Canada) supplement with HEPES (25 mM) and antibiotic-antimycotic (1X) (Invitrogen).

4.3.3 In Vitro Maturation (IVM)

The maturation media was prepared without LH (TCM-199, 0.1% polyvinyl alcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, antibiotics 1X (Invitrogen), 10 ng/ml EGF, 0.57 mM L-cysteine, 0.01 U/ml Folltropin® (Bioniche, Oakville, Canada) for the first 22 hrs of IVM. During this period, one of four treatments was added to maturation media: 1) porcine follicular fluid (Pff, 10%) as control group; 2) NPPC (1000 nM) plus E (100 ng/ml); 3) dbcAMP (1mM);or 4) a combination of the previous two treatments (NPPC+E+cAMP). After the first 22 hrs of

incubation, the COCs were washed and transferred into maturation media with LH (0.01 U/ml, Lutropin - V®; Bioniche) without any treatments. Finally, the COCs were transferred to the maturation media and incubated for another 22 hrs in a 5% CO_2, 5% O_2 and 90% N_2 in a humidified environment at a temperature of 38.5°C. The control group was maintained in the presence of Pff for the next 22 hrs (standard IVP procedure). At the end of IVM, the oocytes were mechanically denuded of cumulus cells using 0.1% hyaluronidase. The oocytes were then washed three times in $MgCl_2$-free phosphate buffered saline (PBS). Immediately after washing, the oocytes (N =70/ replicate) were transferred into a minimal volume of PBS containing a lysis buffer obtained from the Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems, Burlington, Canada). Then, samples were plunged in liquid nitrogen and stored at -80°C until RNA extraction. In each IVM run, oocytes were divided between all treatments.

4.3.4 RNA extraction and amplification

Total RNA was extracted (Six biological replicates for each treatment) using the Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. For high-quality total RNA, the purification procedure was included DNase treatment by using RNase-Free DNase Set (Qiagen, Bay St, Canada) to remove the DNA from samples. Finally, the pure RNA was eluted in 12µl with an elution buffer. For the quality of purified RNA, all sample were evaluated by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA) by using the RNA 6000 Pico LabChip (Agilent Technologies). Only samples that had an RNA integrity number (RIN) of more than 6 were selected (accepted samples: Pff =4, NE =3, CANE =4) for further amplification and microarray process.

The RNA amplification was run using the Arcturus® RiboAmp® HS-PLUS RNA Amplification Kit (Applied Biosystems). This procedure consisted of two rounds of amplification according to the manufacturer's protocol. For the linear amplification process, three nanograms of purified RNA were used in a 6 hrs round of T7 RNA polymerase following manufacturer's directions. Briefly, the purified aRNA went through two rounds of amplification with each round consisting of the following steps: cDNA synthesis, cDNA purification, *in vitro* transcription and aRNA purification. At the end of the second round of amplification, the quantity and the purity of the antisense RNA (aRNA) were measured using NanoDrop™ 2000 (Thermo Scientific, Grand Island, USA). Also, all aRNA samples were analyzed using the 2200 Tape Station (Agilent

Technologies, Santa Clara, USA) for quality control. aRNA samples that showed single, broad peak with similar size in length were selected for further analysis. This step was recommended by the manufacturer's instructions to have confidence that selected samples were homogeneous and the size of aRNA ranged in length from 200-2000 bases. The aRNA for all samples were stored at -80ºC.

4.3.5 Microarray experimental design

This experiment was designed to evaluate the effect of delaying meiotic resumption during IVM using one of three treatments (cAMP (CA), NPPC+E (NE), and NPPC+E+cAMP (CANE)) versus using Pff (standard procedure for IVM) on gene expression of matured oocytes. The global transcriptomic profile of treatments versus Pff was performed using the EmbryoGENE Porcine Array Version1 (EMPV1, Agilent custom array, NCBI GEO platform GPL14925) [361]. The EMPV1 microarray contained a total of 43,675 oligonucleotide probes. Each sample was composed of a pool of matured oocytes (approximately 70 oocytes). After RNA extraction and amplification, three biological replicates that passed a quality control were used for each group (treatment vs. Pff), and the hybridization design was based on the dye-swap design [373]. Each biological replicate was stained during labeling with both Cy3 and Cy5 dye and swapped (6 arrays used per comparison).

4.3.6 aRNA labeling, hybridization and scanning

The aRNA (2 µg) was labeled with the ULS^{TM} Fluorescent Labeling KIT for Agilent arrays using cyanine 3 (Cy3) and cyanine 5 (Cy5) (Kreatech Biotechnology, Amsterdam, Netherlands). The procedure was also included purification step using PicoPure^{TM} RNA Isolation Kit (Applied Biosystems) to remove unlabelled aRNA. By the end of labeling, the concentration of aRNA and dye were measured using NanoDrop 2000 to evaluate the aRNA labeling efficiency. For successful hybridization, the labeling efficiency needs to be in minimum of 30 pmol/µg of labeling signal.

Before the hybridization, the fragmentation of labeling aRNA was run by mixing 825 ng of Cy3 and Cy5 labelled of amplified aRNA, Tomato and Agilent spikes, nuclease-free water plus 25X Fragmentation Buffer, and 10X blocking agent (Agilent Technologies). The fragmentation mix

(55 µl) was incubated at 60ºC for 15 min and cooled on ice for one min. To stop the fragmentation reaction, an equal volume of 2x GEx Hybridization Buffer HI-RPM (Agilent Technologies, Santa Clara, USA) was added to fragmentation mix (total 110µl). This final mix was loaded into each array of EMPV1 microarray (4 x 44K slide, Agilent). The microarray slide was hybridized and washed following the protocol described by Tsoi *et al*. [361].
The microarray slides were scanned using GenePix® 4200 AL Microarray Scanner (635nm for Cy5 and 532 nm for Cy3) with Autoloader (Molecular Device, Sunnyvale, USA). The auto scan image was analyzed using GenePix Pro 4.0 software. Any spots (set with pixel size of 5µm) of the array with blemishes were manually flagged for exclusion in further analysis. The data for analyzed was saved in GenePix Results (GPR) format for further array analysis.

4.3.7 Microarray statistical analysis

Signal intensity data (median intensity value) was analyzed using the FlexArray software version 1.6.3 [374], which is based on the Linear Models for Microarray (limma) Bioconductor package [375]. Data intensity was uploaded to FlexArray as a median. For background correction, the subtraction method (simple) was performed by replacing negative value with 0.5 to avoid 0 or the negative values that can interfere with the log transformation performed during the analysis. For normalization, Loess within-array and quantile between arrays methods were applied. Limma data analysis was run to estimate fold changes of the treatments, which were compared to the control (Pff) using fold change value of ≥ 2 and a *P*-value of ≤ 0.05 as a threshold. The data of three comparisons (CA, NE, and CANE) was analyzed using the False Discovery Rate (FDR) method and no genes were found differentially expressed after application of the FDR correction.

4.3.8 Gene functions

The list of genes that were differently expressed was imported into the Expression Analysis Systemic Explorer (EASE; http://david.niaid.nih.gov/david/ease/help.htm) to identify gene ontology categories that are overrepresented in annotation classes [376]. The statistical analysis for over-represented goups was performed based on EASE score (Modified Fisher Exact Test) and Bonferrroni correction. Categories with an EASE score of less than 0.05 were considered significant. Also, the pathway analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7; https://david.ncifcrf.gov/)) tools

[377]. This program used an algorithm to measure relationships among the annotation (DAVID default population background) terms based on the degrees of their co-association genes, and to group the similar annotation contents from the same or different resources into clusters. Also, the PANTHER 9.0 program (http://pantherdb.org) was used to identify functional annotations using overrepresentation statistical analysis [378]. In these programs, data were different at a *P* value ≤ 0.05.

4.3.9 Quantitative-PCR (qPCR) analysis

For microarray validation, total RNA were taken from three biological replicates of each treatment (non-amplified original materials) and converted to cDNA with ReadyScript ®cDNA Synthesis Mix with RNase inhibitor (Ambion, Burlington, Canada) according to the manufacturer's recommendations. Based on literature review, qPCR was performed on 10 genes (Table 4.5) having a significant differential expression in treated-oocytes. The sequences for these selected genes were retrieved from FASTA in NCBI. Primers for selected genes were designed using the NCBI's primer-blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and BLAST analysis was performed using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Selected primers had to meet the following criteria: primer designed to include primer span exon-exon junction; base length of: 20-24 base; CG content of: 50-60%; annealing temperature of: 55-65ºC; and amplicon size of: 80-150 bp. These primers were commercially synthesized by Integrated DNA Technologies Inc. (IDT, Coralville, IA, USA). The qPCR was performed in Rotor-Gene Q (Qiagen,Valencia,USA) using a Rotor-Gene SYBR Green RT-PCR Kit (Qiagen). The standard curve of each gene was evaluated using five serial dilutions of cDNA. The gene expression was normalized using three reference genes (PPIA, GUSB, PRL15). These genes have been commonly used as reference genes in different studies [379, 380]. In our microarray data, these reference genes also did not differ in our treatment groups. The gene expression was calculated based on the comparative CT method (ΔΔCT) [381] . The statistical analysis of different genes in qPCR was run using a Kruskal-Wallis Rank Sum Test by comparing the values of specific genes in treatment versus Pff (control) using a *P* value ≤ 0.05.

4.4 Results

4.4.1 Gene expression profile analysis of different treatments

A total of 55, 120, and 167 genes were differently expressed in CA, NE, and CANE treatment, groups respectively, compared to the control group (Pff), (fold change ≥ 2 and *P* value ≤ 0.05; Fig. 4.1-A). The number down-regulated genes were 14, 36, and 112 genes, while the up-regulated genes were 41, 84, and 55 genes for CA, NE, and CANE, respectively. The three treatments had only one gene in common (5.8S ribosomal RNA gene; down- regulate in all treatments). Between treatments, the number of genes found to be in common was CA vs CANE =3, NE vs CANE =5, CA vs NE =25 (Fig. 4.1).

4.4.2 Gene ontology and pathway analysis

The EASE program classified the genes into different ontology categories (Table 4.1). The up-regulated genes in the CA group (Table 4.1) highlighted the effect of cAMP on the signal transduction (ARHGAP9, RGS5, BRAO, STAT2, and STAT4), chromosome segregation (TTK and RFC3), and mitochondrial activity (TIMM9, MRPS30, MRPL3, and IDH3B). With the NE treatment, the results indicated that mostly genes related to nucleus metabolism were up-regulated. Some of these genes were related to MAPK pathway activity (MAX, MAPK9, REL) or the ERK pathway (HAT1). The CANE treatment affected genes that regulate metabolism (APOA1, APOD, ACO2, CKMT2, DNAH14, and NDUFB7). This NE treatment also enhanced the pathways involved in growth and cell survival by up-regulating IGF1 and ID4 genes. The DAVID program was used to identify the most significant genes involved in different pathways (Table 4.2). It was observed that the CA treatment significantly up-regulated JAK/STAT and interleukin pathways (STAT2 and STAT4 genes) and the HSP90AA1 gene. In the NE treatment, many genes were involved in the metabolic processes (PPARG, IDH3B, and SETDB1) or cell cycles (CCNB2 and CDK1), while the CANE treatment up-regulated genes in growth (IGF1 and ID4) and metabolic processes (ACO2, APOA1, and CKMT2).

On the other hand, delaying the meiotic resumption strategy down-regulate genes in different treatments (Table 4.3). The NE treatment down-regulated genes involved in fatty acid (SCD5), lipid (PLA2G7), and tyrosine (MIF) metabolisms or ATP synthesis (ATP5B). Only the

interleukin 18 gene was down-regulated by CA treatment. Meanwhile, CANE treatment suppressed genes that had roles in signal transduction pathways (IL6, HBEGF, and TLR7)
The PANTHER program was used to understand the pathways that were over-represented in each treatment (Table 4.4). This analysis showed that CA treatments up-regulated pathways related to signal transduction (JAK/STAT, interleukin, and PDGF pathways), while NE treatment up-regulated genes more related to cell growth and survival. For CANE, up-regulated the pathways involved in the mannose metabolism, TCA cycle and integrin signaling. Also, the cytoskeleton regulation pathway affecting the intracellular cytoskeleton in the cell was also up-regulated.

4.4.3 qPCR validation

PCR validation failed to demonstrate significant differences in the gene expression between microarray and qPCR analytical tool (Fig. 4.2). Most trends in expression genes in qPCR followed the same pattern of microarray except for two genes: RGS5 (Incresed in microarray vs decreased in qPCR) .and SCD5 (Decreased in Microarray vs increased in qPCR).

Table 4.1: The EASE analysis results of up-regulated genes from different groups that involve in different ontology categories.

	System	Gene Category	EASE score	Genes
CA vs Pff	**GO Cellular Component**	cytoplasm	1.72E-02	BRAP; CHIC2; IDH3B; MRPL3; MRPS30; RFC3; RGS5; STAT2; STAT4; TIMM9; TTK
	GO Molecular Function	enzyme activator activity	2.31E-02	ARHGAP9; RFC3; RGS5
	GO Biological Process	JAK-STAT cascade	3.48E-02	STAT2; STAT4
NE vs Pff	**GO Biological Process**	DNA metabolism	2.29E-03	ASF1A; CRY1; HAT1; PMS1; SETDB1; SPO11; UBE2A
	GO Cellular Component	nucleus	2.45E-03	ASF1A; CCNB2; CFL2; HAT1; MAPK9; MAX; PMS1; PPARG; PSMD5; REL; SETDB1; SPO11; TAF13; TRIP4; UBE2A; ZNF277
	GO Biological Process	nucleotide and nucleic acid metabolism	1.26E-02	ASF1A; CRY1; HAT1; MAX; PAICS; PMS1; PPARG; REL; SETDB1; SPO11; TAF13; TRIP4; UBE2A; ZNF277
CANE vs Pff	**GO Molecular Function**	high-density lipoprotein binding	1.73E-02	APOA1; APOD
	GO Cellular Component	cytoplasm	1.97E-02	ACO2; ACTA1; ACTA2; AP2B1; APEH; CKMT2; DAP3; DNAH14; DTNB; EXTL2; GMDS; ITGA6; NDUFA9; NDUFB7; PDLIM1; PLP2; PRKRA; RGS5; RPS8; S100A12; SNX3
	GO Biological Process	cell motility	2.33E-02	AAMP; ACTA1; CKMT2; FN1; IGF1

Table 4.2: The functional pathway analysis from DAVID program showing significantly up-regulated genes involved in pathways associated with different treatment groups.

	Gene names (log_2 fold change)	Pathways		Gene names (log_2 fold change)	Pathways		Gene names (log_2 fold change)	Pathways
NE vs Pff	**SETDB1(1.36)**	Lysine degradation	**CA vs Pff**	**IDH3B (1.03)**	Citrate cycle(TCA cycle)	**CANE vs Pff**	**ACO2 (1.04)**	1-Citrate cycle (TCA cycle), Glyoxylate and dicarboxylate metabolism
	CDK1 (1.08)	Cell cycle, Oocyte meiosis, p53 signaling pathway, Gap junction, Progesterone-mediated oocyte maturation		**HSP90-AA1 (1.05)**	Antigen processing and presentation, NOD-like receptor signaling pathway, Progesterone-mediated oocyte maturation, pathways in cancer, Prostate cancer		**APOA1 (1.82)**	PPAR signaling pathway
	CFL2 (1.18)	Lysine degradation					**CKMT2(1.01)**	Arginine and proline metabolism
	CCNB2 (1.24)	Cell cycle, Oocyte meiosis , p53 signaling pathway, Progesterone-mediated oocyte maturation		**STAT2 (1.11)**	Chemokine signaling pathway, Jak-STAT signaling pathway		**ID4 (1.01)**	TGF-beta signaling pathway
	IDH3B (1.10)	Citrate cycle		**STAT4 (1.12)**	Jak-STAT signaling pathway		**IGF1 (1.91)**	Oocyte meiosis, p53 signaling pathway, mTOR signaling pathway, Focal adhesion, Long-term depression, Progesterone-mediated oocyte maturation, Aldosterone-regulated sodium reabsorption Pathways in cancer, Glioma, Prostate cancer, Melanoma, Hypertrophic cardiomyopathy (HCM), Dilated cardiomyopathy
	PPARG (1.02)	PPAR signaling pathway, Huntington's disease, Pathways in cancer, Thyroid cancer,						

Table 4.3: The functional pathway analysis from the DAVID program showing significantly down-regulate genes involved in pathways associated of different treatment groups.

	Gene names (log_2 fold change)	Pathways		Gene names (log_2 fold change)	Pathways		Gene names (log_2 fold change)	Pathways
NE vs Pff	**IL18 (-1.31)**	Cytokine-cytokine receptor interaction, NOD-like receptor signaling pathway, Cytosolic DNA-sensing pathway	**CA vs Pff**	**IL18 (-1.02)**	Cytokine-cytokine receptor interaction, NOD-like receptor signaling pathway, Cytosolic DNA-sensing pathway	**CANE vs Pff**	**IL6 (-1.18)**	Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, Cytosolic DNA-sensing pathway, Jak-STAT signaling pathway, Hypertrophic cardiomyopathy (HCM)
	MIF (-1.75)	Tyrosine metabolism, Phenylalanine metabolism					**HBEGF (-1.24)**	ErbB signaling pathway, GnRH signaling pathway
							BLM (-1.17)	Homologous recombination,
	PLA2-G7 (-1.99)	Ether lipid metabolism					**PPP1CB (-1.25)**	Oocyte meiosis, Vascular smooth muscle contraction, Focal adhesion, Long-term potentiation, Regulation of actin
	PSME2 (-1.16)	Proteasome Antigen, processing and presentation					**TLR7 (-2.27)**	Toll-like receptor signaling pathway,
	SCD5 (-1.03)	Biosynthesis of unsaturated fatty acids, PPAR signaling pathway						

Table 4.4: Results of pathway analysis of gene expression in matured oocytes in different treatment groups. Statistical overrepresentation test was used in the PANTHER program to identify the most significant pathways from the gene list.

		Pathway	genes	*P*-Value
Up-regulated genes	**NE**	Circadian clock system	CRY1	2.39E-02
		Oxidative stress response	MAX, MAPK9	2.47E-03
		Toll receptor signaling	REL, MAPK9	1.01E-02
		Ubiquitin proteasome	UBE2A,UBA2T	1.27E-02
		Apoptosis signaling	REK,MAPK9	3.86E-02
		EGF receptor signaling	MAX,DAB21P	4.81E-02
	CA	JAK/STAT signaling	STAT2, STAT4	4.97E-04
		Interleukin signaling	STAT2,STAT4, BRAF	3.46E-04
		Ras signaling	STAT4, BRAF	5.27E-03
		PDGF signaling	STAT2,STAT4, BRAF	9.09E-04
	CANE	EGF receptor signaling	STAT2, STAT4	1.41E-02
		Mannose metabolism	GMDS	1.63E-02
		TCA cycle	ACO2	2.43E-02
		Integrin signaling	FN1, ITGA6	
		Cytoskeletal regulation by Rho GTPase	ACTA1, ACTA2	4.03E-02
Down-regulated genes	**NE**	ATP synthesis	ATP5B	1.62E-02
		Endogenous_cannabinoid signaling	GNG11	2.3E-02
	CA	Toll receptor signaling	IL18	2.71E-02
	CANE	Asparagine and aspartate biosynthesis	ASNS	2.80E-02
		Coenzyme A biosynthesis	PANK3	4.36E-02

Table 4.5: Oligonucleotide primer sequences that were used for real-time qPCR analysis to validate microarray results

Gene	Accession#	Oligo	Primer sequence	Amplicon size (bp)	Annealing temperature(ºC)
STAT4	NM_001973051	Forward Reverse	CATGTATGCCAACCCACCCT TGCTGAGAGTTGAAACATTCTTGT	150	60.03 59.12
BRAF	XM_003134610.3	Forward Reverse	AACTTGATTTGCTGTTTGTCTCCA AGGCTGTGGGATTGGAATGG	182	59.60 60.00
HSP90AA1	NM_213973.1	Forward Reverse	TGTCAGTCACCAAAGAGGGC CGACACAACCACCTTTTCGAC	143	59.90 60.00
MRPL3	XM_001925038.3	Forward Reverse	CAGGAACTTGGATTGCCTCC ATAGAGGGGTGCCTGGCTTA	85	58.53 60.03
CDK1	NM_001159304.2	Forward Reverse	AAGCTGGGATCTACCACATCCA CATGGCTACCACTTGACCTGT	128	60.90 60.00
SCD5	NM_001114278.1	Forward Reverse	CACCGTCTCACTCAACGTCA TTGTGGAAGCCTTCACCGAT	135	59,97 59.60
PPARG	NM_214379.1	Forward Reverse	TCCATGCTGTCATGGGTGAA ACCATGGTCACCTCTTGTGA	103	59.05 60.25
RGS5	NM_214356.1	Forward Reverse	CGGAGGCTCCTAAAGAGGTGA GAAGCGAGGCAGTGAATCCT	143	60.96 60.11
IGF1	NM_214256.1	Forward Reverse	GCCCAAGGCTCAGAAGGAAG GAGCAAAGGATCCTGCCAGT	130	60.68 60.03
ACO2	NM_213954.1	Forward Reverse	CGAGTAGGTCTGATCGGCAG GAGCCCGGTGTGATGGTAAA	128	59.69 60.04
PPP1CB	NM_214184.2	Forward Reverse	TCTGTTGTCACGGAGGACTGT CCCCAACCTTGCACATCCTTA	142	61.03 60.27

Fig. 4.1: Venn diagram summarizing number of known genes after microarray analysis of porcine matured oocytes that were delayed meiotic resumption using different treatments during in *vitro* maturation in comparison to the Pff group (control). Up- and down-regulated of known genes were determined using fold change of ≥ 2 and $P \leq 0.05$.

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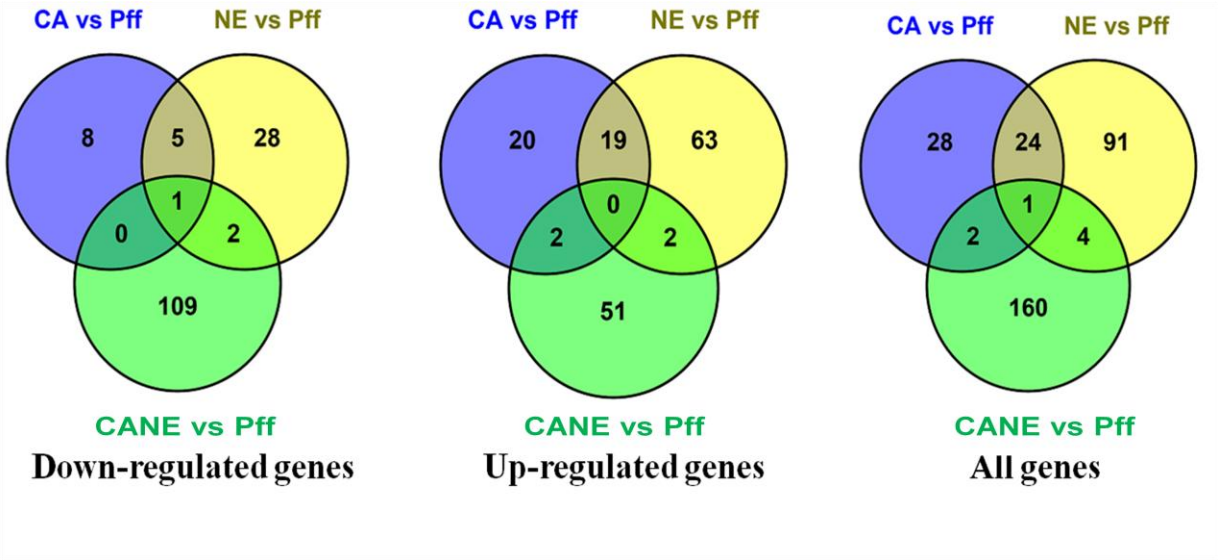
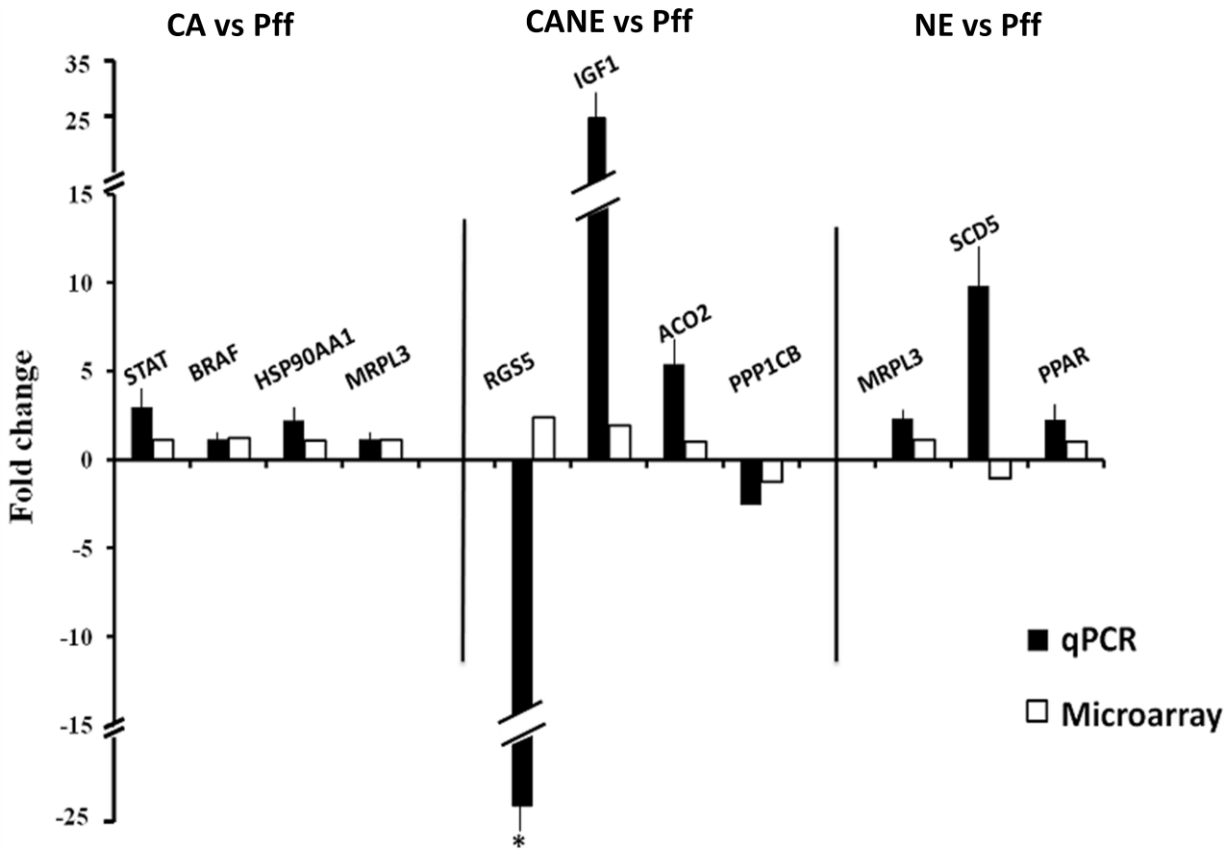


Fig. 4.2: The expression of different genes (log of fold change) from matured oocytes after delaying meiotic resumption during IVM using qPCR (three biologic replicates /for each treatment). Solid bars represent the expression (± SEM) of genes by qPCR. The open bars represent the expression of the same group of genes measured by microarray. *The value is significantly different ($P \leq 0.05$) in treatment groups compared to control



4.5 Discussion

The present study was to identify the effect of delaying meiotic resumption protocol after IVM on gene profile of porcine oocytes in compared to using standard procedures (Pff). It could be expected that CANE treatment would have many differentially expressed genes and pathways in common with the CA and/or NE treatments because CANE was a combination of CA and NE treatments. However, the results showed a very limited number of differentially genes in common between CANE and other treatments (Fig. 4.1). Previous studies have reported that the interaction of estradiol with dbcAMP can affect gene expression regulation differently [382-384]. Estradiol mediates its effect in oocytes through receptors that modify gene transcription [385]. However, it was observed that PKA stimulated by cAMP can modulate estradiol receptor (ER) response through phosphorylating the ER residues [382]. Therefore, both estradiol or cAMP can modulate the ER response differently in term of gene expression pattern [383]. All steroid receptors including ER are ligand-activated transcription factors that can be activated by non-steroid hormones [386]. The ability of cAMP to elicit ER response is a ligand-independent manner that can lead to specific gene expression response [383]. As an example of this different interaction, a study investigating the expression of estrogen receptors (ESR2a and IHCGR) in ovarian follicles of zebrafish when treated with dbcAMP (1 mM) and estradiol (50 nM) [387]. Treated oocytes had different gene expressions in comparison of the use of both substances individually. These findings could explain the limited number of common genes between CANE and other treatments for our study (Fig. 4.1). The use of threshold (two-fold) to establish a list differentially expressed gene list could be another explanation for the few number of common genes between CANE and other treatments. Using 1.5 fold changes, our analyses revealed more common genes between CANE and other treatments (data not shown). However, the number of common genes discovered with a lower cut-off was still limited. Regarding the common genes between the other groups, it was observed that CA and NE treatments had 25 common genes, which represent 42 % or 19% of the total population of differentially expressed genes, respectively. Except for MRPL3, most of these common genes did not show any direct effects on oocytes competence. MRPL3 was found to be associated with an increase of oocyte competence, by influencing the transcription of other genes involved in oocyte development [388]. This conclusion was established based on many previous microarray studies that compared matured oocytes under *in vitro* and *in vivo* conditions for bovine and mouse [389]. Our data clearly has

shown that each treatment used to delay meiotic resumption has a unique impact on the molecular pathway to improve the oocytes competence.

The dbcAMP that used to delay meiotic resumption of cattle and pigs oocytes [10, 359] has shown increasing the number of *in vitro* produced embryos. Our study has shown that treating the oocyte with CA during *in vitro* maturation (first 22 hrs), resulted in up–regulation of different pathways related to an increase of the mitogen-activated protein kinase (MAPK) pathway activity (Table 4.4). The Ras, JAK/STAT and PDGF pathways were up-regulated and found to be involved in MAPK pathways. These pathways consist mainly of three genes (STAT2, STAT 4 and BRAF); these genes are involved in MAPK activities [390]. This result supports previous studies [227, 391] that using dbcAMP to delay nuclear maturation for 22 hrs will increase mitogen-activated protein kinase (MAPK) in pig oocytes by 44 hrs of IVM, and modulated the intracellular signal pathways in responses extracellular signals [392, 393]. This transduction signal induces an activation of PI3 kinases (phosphatidylinositide 3-kinases), which are usually involved in cellular proliferation, development, and activation of the anti-apoptotic process in oocytes [390, 394]. Therefore, up-regulated genes that are involved in MAPK activity can reflect positively in our study of oocytes by up-regulating genes such as the heat shock protein 90 (HSP90AA1) and mitochondrial ribosomal protein L3 (MRPL13). These genes play a role in oocytes competence [389]. Inhibition of HSP90AA1 during IVM reduced the developmental competence of bovine oocytes and negatively affected the blastocyst rate [395]. Also, MRPL3 is considered a gene marker for oocyte competence [388], and was up-regulated in our study. Thus, the elevation of expression of the MRPL3 and HSP90AA1 genes observed in the CA group could suggest that they can support oocyte competence without the presence of Pff.

Treating pig oocytes with NE to delay oocyte meiotic resumption has been shown to up-regulate different MAPK pathway signaling genes (MAX, MAPK9, and REL) [396-398]. The importance of the expression of these genes is related to their roles in meiotic maturation, development of oocyte in mammalian [390, 399]. It has also been established that estradiol can induce a rapid activation of MAPK in mammalian cells with an event that is independent in terms of transcription and protein synthesis [400, 401]. Also, the NE treatment up-regulated the oxidative stress response, which prevents Reactive Oxygen Species (ROS) damage in oocytes during IVM [402]. This response could protect the oocytes from damage and has a positive effect on oocytes development and competence. Therefore, the up-regulation of gene activities induced by NE

treatment could support the growth and development of oocytes, and indirectly support the embryo development.

The CANE treatment significantly up-regulates genes involved in the metabolic process, growth, and cell survival (Tables 4.1, 4.3, 4.5). Interestingly, the CANE treatment significantly increased IGF1 gene expression, which has been found to improve the competence of oocytes in different species including pigs [403] bovine [404] and mice [405]. Increasing this growth factor's expression may play an important role in improving oocyte competence and achieving a successful IVF–IVC process, as we previously demonstrated with a similar blastocyst rate using CANE treatment compared to Pff (Chapter 3).

In general, all treatments used in this study activated many metabolic processes at different levels (Table 4.1, 4.2, and 4.4). During IVM, the oocytes usually go through many intracellular changes toward maturation that require energy consumption [302, 406]. These result are in agreement with a study of mouse oocytes that found the genes controlling metabolism are up-regulated in MII when compared to the GV stage [295]. The metabolic processes associated with oocytes development plays an important role in determining oocyte competence [52].

In summary, this study demonstrated that different methods used to delay meiotic strategy will have their own molecular effect leading to the maturation of pig oocytes. Among the treatments tested in this study, only a few molecular pathways (metabolic process and growth and cellular development) were common among the group. Same phenomenon was observed when we specifically analyzed the gene expression, only one gene was common and highly expressed between the treatments (5.8S ribosomal RNA). Regardless of the strategy used to delay meiotic resumption, it will lead to a same in vitro development of pig embryos (Chapter 3).

CHAPTER 5

COMPARISON BETWEEN A CLOSED AND OPEN DEVICES TO VITRIFY *IN VITRO* PRODUCED PIG EMBRYOS

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Hayder Al-Shanoon: Perform the study, participate in the experimental design, wrote the manuscript

Philippe Godin: Collecting of samples, produced embryos, participate to experimental design, and review of the manuscript

Carl Lessard: Experimental design and critical revision for manuscript

Citation of the paper:

This paper has not been submitted yet

Relationship of this study to the dissertation

In previous studies, we highlighted the importance of the strategy of delaying meiotic resumption in improving the porcine embryo production *in vitro*. Also, the ability to cryopreserve the embryos for both long and short term safely and without contamination has presented another challenge in pig IVP. In this study, I examined two vitrification methods dependent on direct contact with liquid nitrogen (Open–pulled straws, OPS) or no contact (French straw, FS) that reduced the possibility of contamination by liquid nitrogen. The study evaluated the effect of vitrification procedures on embryo quality and viability. We hypothesized that embryos produced *in vitro* would survive the vitrification processes at an equivalent rate whether using a closed (FS) or an opened device (OPS).

5.1 Abstract

Pig embryos have been vitrified successfully after direct exposure of embryos to liquid nitrogen using open pulled straws or cryoloop. Using an open carrier, vitrified pig embryos can be contaminated from pathogenic organisms that could be contained into the liquid nitrogen vessel. This study hypothesized that *in vitro* produced embryos would survive vitrification procedures at an equivalent rate using a closed (French straws (FS)) or an open device (Open Pull Straw (OPS)). *In vitro* derived morulas were produced using standard procedures and vitrified on day 5 of *in vitro* culture (FS; n=127, OPS; n=115). The quality of the embryos (morphological integrity) was evaluated after warming and after 48 hours of culturing. After warming, 85% of vitrified embryos showed a quality of grade 1 for both FS and OPS groups. After 48-hr of culture, morulas did not develop to a blastocyst stage. The number of embryos stained with propidium iodide (PI) increased significantly ($P = 0.02$) with OPS compared to FS. Morulas of quality of grade 1 decreased to 70% for FS group and 63% for OPS group. In conclusion, *in vitro* produced embryos can be efficiently vitrified using a closed system and be stored in biosecure manner enabling distribution or long-term storage using a closed system.

5.2 Introduction

Cryopreservation of pig genetic material (oocytes, semen, and embryos) is a valuable method used to preserve the genetic diversity of pig breeds [407]. Many advantages can be gained from this process, including: the protection of genetic material of a species or breed from the risk of outbreak disease, the reduction in the transportation cost compared to live animals, and applications in both research in bio-medicine and reproductive technologies [313, 408]. Vitrification is a technique that uses a rapid cooling process by direct transfer into liquid nitrogen [305]. Oocytes [409], sperm [410], embryos [411] and reproductive tissues [412] derived from pigs have been successfully preserved using this technique. From a conservation point of view, vitrified embryos are preferable to oocytes or semen due to the ability to preserve the entire genome of an animal. The success in preserving pig embryos in the short- or long-term using vitrification can be considered one of essential elements of an effective embryo transfer industry [281]. Although the embryo transfer in the pig was limited in commercial work for a long time due to unavailability of an acceptable nonsurgical method, successes were reported recently

[413, 414]. Research on vitrification process is important to deliver viable embryo and to be used in embryo transfer whenever it is required.

Successful cryopreservation of porcine embryos, however, faces obstacles due to their high lipid content, which makes them very sensitive to chilling damage [415, 416]. This sensitivity is related to the stage of embryonic development [417]. Blastocysts and morulas have been reported to be less sensitive than earlier 2–4 cell stages, because the lipid content is reduced as embryo development progresses [315]. Vitrification procedures were demonstrated to deliver more viable pig embryos for using in different applications such as embryo transfer and biomedical studies. The reason for successful vitrification of pig embryos is the extreme rate of cooling (up 20,000 ºC/min), which prevents chilling injuries associated with the lipid contents in the cells [308, 418]. Various methods have been used to vitrify embryos, which can be classified into two types based on vitrification device used to load the embryos [313]: open and or closed system devices [419]. In closed system devices such as conventional French straw (FS), embryos have no direct contact with liquid nitrogen (LN_2) and they are frozen at a rate of 2,500ºC/min [420]. On the other hand, open devices such as the Open pulled straw (OPS) allow direct contact of embryos with LN_2 and the embryos are frozen at a rate of 20,000ºC/min [420, 421]. The high cooling rate prevents chilling injury to cells and reduces the exposure time of embryos to cryoprotectants, reducing toxicity effect in embryos [419, 422]. Open system devices have been used successfully to vitrify pig embryos, resulting in live piglets [423-427]. However, there are biosecurity concerns associated with direct exposure of the embryos to LN_2, which can be contaminated by fungal spores, yeasts, bacteria, and viruses [428-430]. Theoretically, the contamination can occur even with sterile LN_2 due to the risk of cross-contamination with infected samples [431]. This contamination risk may limit the vitrification technique from being commercially used in swine industry and it is contrary to the recommended procedures for international distribution (requiring a closed device for the frozen embryos [432]). Many reports have indicated that using sealed carriers during cryopreservation is effective in protecting the germplasm from contamination, compared to the use of open carriers [430, 431, 433, 434]. The goal of this study was to compare the ability of *in vitro* produced embryos at morula stage to survive vitrification procedures using an opened (OPS) or closed (FS) device and to develop normally under *in vitro* conditions. Specifically, we hypothesized that *in vitro* produced morulas will survive the vitrification procedures and develop to blastocyst at the same rate. The objective

of this study was to evaluate the ability of in vitro produced embryos to survive vitrification procedures with a closed or opened carrier.

5 **3 Material and methods**

5.3.1 Chemicals and media

All the chemicals and media were purchased from Sigma Chemical Co, Oakville, Canada ; the exceptions were noted in the following sections.

5.3.2 In vitro production of embryos

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within two hours, in a thermos containing pouches pre-warmed to a temperature of 37ºC. Then, the ovaries were cleaned of adjacent tissues and rinsed in saline (0.9% NaCl) three times at room temperature. The cumulus oocyte complexes (COCs) were aspirated from follicles of 3–6 mm in diameter. Oocytes with a homogeneous cytoplasm and three or more cumulus cell layers were selected for this study and transferred into TCM-199 (Invitrogen, Burlington, Canada) supplemented with HEPES (25 mM) and antibiotic-antimycotic (1X, Invitrogen). COCs were washed three times in this medium, then transferred to a maturation media (TCM-199, composed of 0.1% polyvinyl alcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, antibiotic-antimycotic 1X (Invitrogen), 10 ng/ml EGF, 0.57 mM L-cysteine, 0.01 IU/ml Lutropin-V® (Bioniche, Oakville Canada), 10% of porcine follicular fluid, and 0.01 IU/ml Folltropin® (Bioniche). The COCs were grouped up to a maximum of 50 and incubated for 44 hrs in a 5% CO_2, 5% O_2, and 90% N_2 in a humidified environment at 38.5°C.

Matured oocytes were washed three times in a modified tris-buffered medium (mTBM) supplemented with 1 mM caffeine and 0.4% bovine serum albumin (BSA; Minitube,Verona, USA) [435]. For semen preparation, frozen semen straws were thawed at 50°C for 10 seconds, diluted with mTBM media, and centrifuged twice at 1000X for 7 min. The concentration of sperm added to a group of 50 oocytes was 0.1×10^6 sperm/mL. After incubation of the matured oocytes with sperm for 6 hrs at 38°C (in an environment of 5% CO_2, 5% O_2 and 90% N_2), the presumptive zygotes were washed and cultured in NCSU-23 (Zenith Biotech, Guelph, Canada) supplemented with 0.4% BSA. These zygotes were incubated in 5% CO_2, 5% O_2 and 90% N_2 in a humidified environment at 38°C for 5 days.

5.3.3 Vitrification and warming of embryos

Only morulas of grade 1 were selected for the vitrification procedures (see section 5.3.3 for a definition of embryo grade). Two different devices were used: French straw and open pulled straw. All the manipulations were performed at room temperature.

5.3.3.1 French straw (FS) method:

Embryos were vitrified using the French straw (0.25 cc; IVM Technology (Guelph, Canada)). The Syngro Bovine Vitrification Kit (Bioniche, Pullman, USA) was used according the manufacturer's instructions. Briefly, the embryos were immersed in two vitrification media: VS1 and VS2, for 5min and 40–60 sec respectively. While the embryo was placed in VS1, the straw was loaded with 95 µl of diluent and filled with an air column of 10 mm. After the embryo was transferred from VS1 to VS2, the embryo and a small volume of VS2 solution were drawn into the straw. A third column of air was made and the final column of diluent completed the filling of the straw before being sealed ultrasonically. Afterward, the straws were suspended for 1 min in a container cooled down by the LN_2 and the cooled straws were directly plunged into liquid nitrogen. These straws were stored in LN_2 for a minimum of 2 weeks.

For the warming procedure, the straw was removed from LN_2, left at room temperature for 10 sec and then placed in warm water (35ºC) for 20 sec. After warming, the content of the straws was mixed by flicking (5-7 times) and the straws were incubated in a water bath at 22–25^{o}C for 5 min prior to wash (3x) and transfer the embryos to a culture media 48 hrs.

5.3.3.2 Open pulled straws (OPS) method:

The protocol for OPS was followed according to the manufacturer's recommendations (Minitube International, Delavan, USA). Three types of media were prepared during the OPS vitrification procedure. The first was the holding media (HD) composed of a PBS (X1) supplement with 0.5% BSA (w/v). The second was the OPS vitrification solution I (OPS-I) contained HD media supplemented with 7.5% (v/v) of ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO). The third media (OPS-II) was a sucrose media (SM, 1M of sucrose dissolved in HD) supplied with 16.5% (v/v) of EG and 16.5% (v/v) DMSO.

Around 3–5 embryos at morula stage were transferred into HD media for 1 min and moved to another drop of HD. These embryos were placed in OPS-I and subsequently transferred into a small drop (approximately 20 µl) of OPS-II. After a few seconds, the embryos were pipetted

with a small amount of media to create a drop of 2 µl. Then, the thin side of the OPS straw was used to touch the small drop from the left side at a 30^{o} angle. The embryos transferred into OPS by capillary action. Immediately afterward, the OPS were plunged into LN_2 horizontally. Then, the straws were directly stored in a small holder in LN_2 for at least 2 weeks.

For the warming procedure, HD and sucrose media (SM) (holding media supplement with 1M of sucrose) were prepared and all media warmed to 37ºC. After the OPS was taken out from LN_2, it was held with thumb and middle finger for 3 sec. Then, the OPS from thin part was immersed in mixture of HD and SM with a ratio of 2:1(v/v) in 45º angle. Immediately after the vitrified solution column melts, the mixture HD: MS media started diffused into the straw. By closing the open end with index a finger, the solution that melted containing the embryo flowed in HD: MS media. Immediately, the embryos were transferred to another drop with the same mixture for 5 min. After that, the embryos were transferred to a mixture of HD and SM with a ratio of 4:1 for 5 min. The last transfer for the embryos was made into HD media. Finally, the embryos were washed in a 3X culture media (described above) before being observed for quality and incubated for 48 hrs in NCSU-23.

5.3.4 Evaluation of Embryo Quality

The quality of the embryos was evaluated based on morphological integrity as described by Bó and Mapletoft [336]. All selected embryos for vitrification had a quality of grade 1. After warming, the quality of embryos was evaluated and these embryos were transferred into NCSU-23 for 48 hrs. Embryos were classified into three categories: Grade 1 (G1) had a symmetrical and spherical embryo mass with individual blastomeres; Grade 2 (G2) had moderate irregularities in the overall shape of embryonic mass or in size, with at least 50% of the cellular material in an intact, viable embryonic mass; Grade 3 (G3) had the most embryonic mass irregularities in the overall shape, with at least 25% of the cellular material in an intact, viable embryonic mass (Fig.5.1).

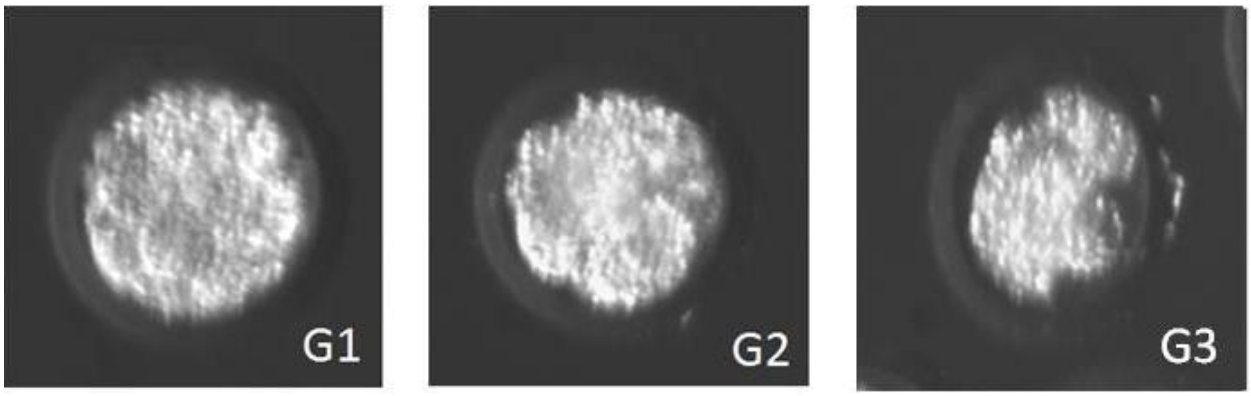

Fig. 5.1: Examples of classifications of post-warmed embryos after 48 hrs of culture. The embryos were classified into different grades as follows: Grade 1 (G1) has a symmetrical and spherical embryo mass with individual blastomeres; Grade 2 (G2) has moderate irregularities in overall shape of embryonic mass or in size, with at least 50% of cellular materials in an intact, viable embryonic mass; Grade 3 (G3) has major irregularities in the overall shape of embryonic mass or size, with at least 25% of cellular material in an intact, viable embryonic mass.

5.3.5 Evaluation of Embryo Viability

The viability assessment was performed by evaluating cell permeability to propidium iodide (PI) [400]. At the end of the culture, the embryos were washed three times in PBS 1X supplemented with 1% PVA. After washing, the embryos were transferred to a staining solution (washing buffer supplemented with 12 µM of PI) and incubated for 7-8 min at 37^{o}C in the dark. Then, the embryos were washed again five times in washing buffer. Each morula was observed under fluorescent microscopy to assess the number of cells stained with PI. They were then classified into five categories depending on the number of cells stained: 0, 1-2, 3-4, 5–7, > 8 cells.

5.3.6 Statistical Analysis

Statistical analysis was performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). The effect of vitrified procedures on the number of fluorescent spots in embryos and the quality of embryos were analyzed using a chi square method followed by Fisher's least significant difference (LSD) test. A P-value ≤ 0.05 was considered significant

5.4 Results

5.4.1 Quality of Embryos

In total, 242 embryos were *in vitro* produced and vitrified using OPS (n=115) or FS (n=127) devices. After vitrification and warming procedures, no differences in the quality of the embryos were found between the devices. Around 85% of the morulas maintaining the quality of G1 (Table 5.1). After 48 hrs of culture, morulas did not progress to a blastocyst stage (data not shown). The number of morulas of G3 increased and was significantly different between the carrier devices used for vitrification ($P = 0.02$). Also, the number of embryos of G1 was reduced when compared to after warming (FS, $P = 0.004$ and OPS , $P < 0.001$). This reduction of grade 1 quality between 0 and 48 hrs exhibited the same pattern in both methods.

		0 hr			48hr		
		embryo quality			embryo quality		
		G1	G2	G3	G1	G2	G3
Treatments	FS (n=127)	108[1]	19	0	88 [2]	33	6[b]
	OPS (n=115)	100[1]	14	1	72 [2]	28	15[a]

Table 5.1: Effect of vitrification-warming procedures using FS (French straw) or OPS (Open–Pulled Straws) devices on the quality of morulas after warming (0 hr) and after culturing (48 hrs). Morulas were classified based on their quality: grade 1 (G1), grade 2 (G2), grade 3 (G3). Values with different letter or number were significantly different ($P \leq 0.05$). The small letter shows the difference within the grade, the number shows the difference between 0 and 48hrs in G1

5.4.2 Permeability of the Cells to PI

After 48 hrs of culture, a majority of the morulas (over 70%) had 2 or lower number of cells stained with PI (Fig. 5.2). Interestingly, embryos vitrified with FS device had greater proportion of embryos without fluorescent red spots ($P = 0.03$) compare to OPS device. Also, the morulas vitrified with OPS had a higher proportion of stained cells (5–7 red spots; $P = 0.02$) than embryos vitrified with FS. No differences were observed with the other categories (1-2, 3-4, > 8 red spots groups) between embryos vitrified with OPS and FS.

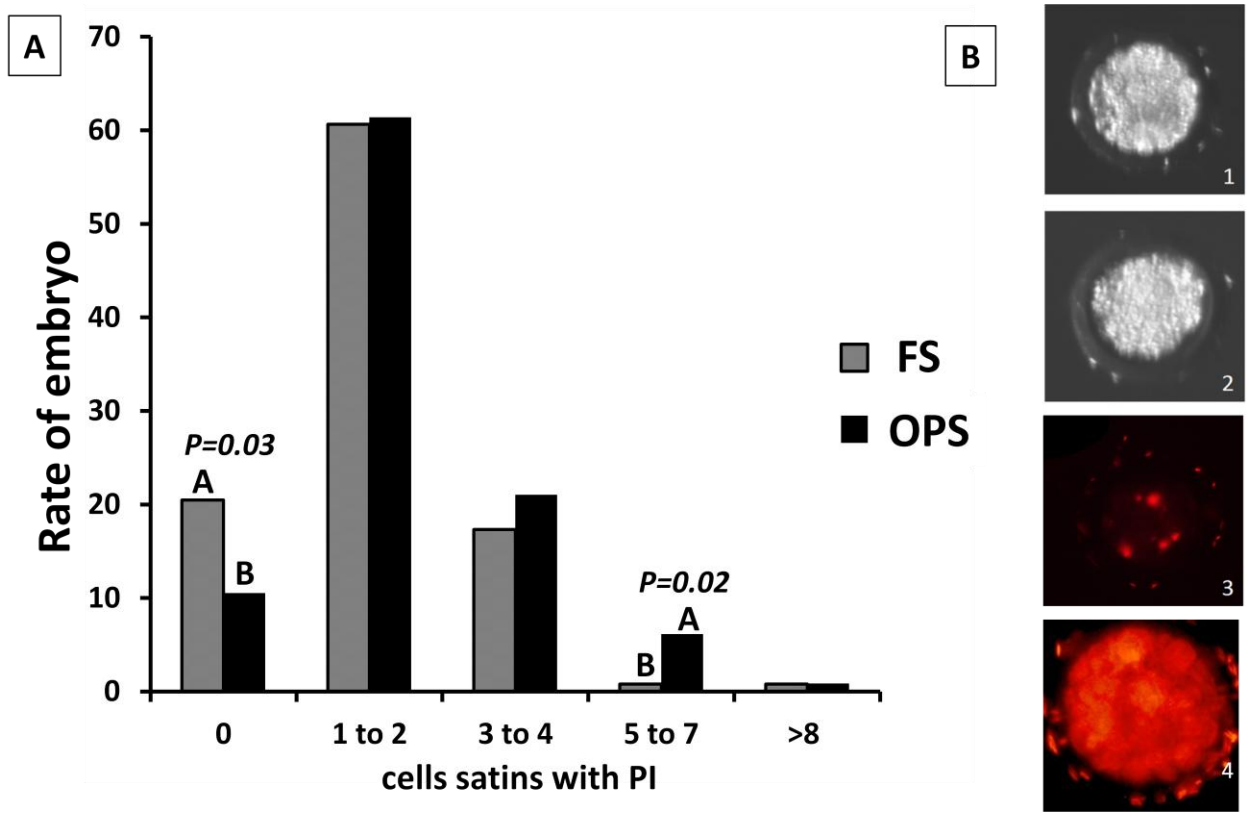

Fig. 5.2: Cellular integrity of vitrified embryo with OPS or FS after warming and culturing for 48 hrs. (A) Proportion of morulas classified based on the number of PI-stained cells: 0, 1-2, 3-4, 5–7, or > 8 cells). (B) Examples of pictures of morula at 0 hr (1), 48 hrs (2), morula after PI staining (3), and dead morula after PI staining (4). Device with different superscript were statistically different ($P \leq 0.05$).

5.5 Discussion

Based on embryo quality (Table 5.1) and cell integrity (Fig. 5.2), this study demonstrated that *in vitro* produced embryos can survive the vitrifying-warming procedures regardless of the device used for vitrification. The majority of the vitrified embryos kept their quality of grade 1 after warming and culturing (Table 5.1). These results suggest that the different vitrification procedures induced minimum structural damages to the embryos. However, *in vitro* produced pig embryos vitrified with OPS had a higher proportion of morula of grade 3 after 48 hrs of culture, suggesting that vitrification using FS could potentially offer a better protection against the cryodamage.

In this study, PI permeability was used as a cell viability assay [436] and it is well known that different cell viability assays could generate different results [437]. Therefore, it is possible that these embryos were more affected by the vitrification processes that we observed, which could explain the arrest in the development for the morulas observed after 48 hrs of culture. However, this observation could also be explained by the suboptimal culture conditions of the *in vitro* production system or the source of oocytes. Suboptimal culture conditions still remain a major factor affecting the yield and quality of pig embryos obtained *in vitro* [438, 439] which is less than the production rate obtained in the bovine species. The average blastocyst formation derived from IVP in pigs is around 20% [440] compared in bovine blastocyst rates around 35% [215]. Also, the source of oocytes used in this study could be considered another factor to explain the arrest in development of the embryos. Embryos in this study were derived from oocytes from prepubertal gilts. It has been demonstrated that these oocytes had a lower competency compared to those obtained from sows [441]. This lower competency is reflected by a low embryo development [442] caused by insufficient cytoplasmic maturation or increase of polyspermia [190, 201]. Another factor that could interfere in the development of the morulas could be related to their lipid content [411]. Compared to *in vivo* produced embryos, the lipid content is higher in embryos produced *in vitro* [443]. This amount of lipid can affect the survival rate of an embryo if it is not removed before the freezing procedures [314, 444]. In this study, the lipids were not removed and it could explain the lack of development of morulas to blastocysts after 48 hrs of culture. To determine if the vitrified-warmed morulas are functional, the ultimate evaluation would be to transfer these vitrified-warmed embryos in a recipient and determine the pregnancy rate after a determined period. However, it was not the scope of this study.

There was concern in our design that low cooling rate of close carrier (FS) could affect negatively on embryo quality or cell integrity [445]. However, the results of embryo quality after warming did not show any difference between using closed and opened carriers for vitrification. These results indicated that the range of cooling rates of 2,500 ºC/min for FS and 20,000ºC/min for OPS is equivalently efficient to vitrify the embryos without causing too much damages. Different reports demonstrated that pig embryos of different development stages did not show differences in the embryo survival rate after vitrification and warming [314, 444, 445]. Thus, this study confirms that at least 2,500 ºC/min is sufficient to vitrify *in vitro* produced pig embryos at a morula stage.

In this study, the results of embryo quality and cells permeability show that the using FS had fewer embryos cells in G3 (5-7 positive stained-cells) after 48 hours of culture compared to OPS. However, we considered the efficiency of both procedures is equivalent for two reasons. First, embryos in grade 1 and 2 that represent >85% of total embryos after culture are the suitable quality to be used in embryo transfer. As the results showed (Table 5.1; 48 hrs) these embryos in both grades have no significant different between two vitrification procedures. Second, most embryos in terms of cellular damages were classified under 8 (Fig. 5.2) cells stained in PI, and it is known that morula by day 5 of a culture contain more than 100 cells. Therefore, the cellular integrity of vitrified embryos using different vitrification procedures induced minimum structural damages to the embryos, and produced the same survival rate.

In summary, this study shows that a closed carrier is equivalent to an opened carrier to support the vitrification process for *in vitro* produced pig morula. Thus, we recommend using FS or other closed carrier in their vitrification protocol to store pig embryos in liquid nitrogen on a long-term basis. This recommendation would allow an improvement of the biosecurity of storage of germplasm against potential contamination coming from external sources.

CHAPTER 6

6. GENERAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

6.1 GENERAL DISCUSSION

1- Is the general hypothesis of this study supported?

Results of the different studies examined in this thesis supported the general hypothesis that delaying tmeiotic resumption of pig oocytes during *in vitro* maturation has a positive effect on oocyte competency for fertilization and embryo development. The studies in chapter 3 evaluated the ability of different follicular substances (NPPC, dbcAMP, NO, cGMP, estradiol, testosterone) to delay meiotic resumption of pig oocytes. Three of substances, alone or in combination, were successful (NPPC+estradiol, dbcAMP, NPPC+estradiol+dbcAMP) in delaying meiotic resumption when adding to the maturation media (containing no LH) for the first 22 hrs of the maturation period. These substances were able to inhibit the meiotic resumption of most oocytes (>80%). The use of dbcAMP is a common way of inhibiting meiotic resumption in pig and bovine oocytes [14, 195, 365]; therefore, this group was selected as the control for the different study using a delay strategy. The inhibitory effect of NPPC+estradiol, dbcAMP, and NPPC+estradiol+dbcAMP appeared to be reversible as the oocytes resumed meiosis and completed nuclear maturation after being cultured for another 22 hrs in maturation media with LH. The combinations of NPPC+estradiol or NPPC+estradiol+dbcAMP were found to have the same ability to delay meiotic resumption as dbcAMP to improve oocyte competence in pig [14] and bovine [365]. The benefit of delaying a meiotic resumption strategy is that it increases the time that oocytes maintain communication with cumulus cells [324]. This communication allows the oocytes and cumulus cells to exchange factors such that support oocyte cytoplasmic maturation [48]. Our results on embryo development indicated that delaying the meiotic resumption strategy using different treatments (NPPC+estradiol, dbcAMP, NPPC+estradiol+dbcAMP) were equivalent to using Pff in the maturation media (Table 3.2, Chapter 3). The Pff has been found to provide a superior environment for oocyte maturation to improve its competence [190, 253, 446]. For this reason, it has been widely used with pig oocytes during IVM due to the beneficial effects on embryo development [255, 446]. Thus, this study used Pff as a control for the IVM procedure. Although Pff supports oocyte competence, it

is not acceptable commercially for IVM because it contains many unknown factors that may play a role in the maturation process [447]. This problem may make it difficult to evaluate the metabolic requirements of the oocytes. In addition, collecting Pff from different batches may lead to experimental variability because the Pff components can be variable due to the cyclicity status of the slaughtered pig [448], and age of the donors [441]. There is also a potential risk of Pff introducing contamination into the maturation media [9]. Therefore, using one of the inhibitory treatments (chemical-defined IVM media) to delay meiotic resumption can lead to consistent results by removing the variability induced by Pff. Consequently, delaying the meiotic resumption with a defined medium can help standardize IVM media in pig IVF as only BSA needs to be used in the media. This may lead to a commercial application of this technology benefitting the pig industry.

2- Why do we need to find new substances to delay meiotic resumption when dbcAMP has shown successful results?

The dbcAMP has been used to delay meiotic resumption in many species and has been shown to provide consistently positive results in embryo development [14, 195]. However, the wide range of biologic effects of cAMP [449] makes it hard to determine how this treatment could affect oocytes. It is possible that the positive effects of dbcAMP treatment resulted from its overall biological effects on the cell, not just its role in delaying meiotic resumption. To determine this point, different substances were evaluated to replace dbcAMP, including NO, cGMP, NPPC, progesterone, estradiol and testosterone (Chapter 3). Only NPPC+estradiol and the combination of NPPC+estradiol+dbcAMP demonstrated efficient results in delaying meiotic resumption (Chapter 3).

When NPPC bind to its receptor (NPR2), an elevation in cGMP will stimulate an increase of cAMP concentration, as the dbcAMP will do. For this reason, the NPPC was found to delay meiotic resumption in pig oocytes. However, NPPC has not been studied regarding its effect on cytoplasmic maturation and embryo development in the pig. Therefore, the IVF-IVC was performed using NPPC+E and dbcAMP treatments to evaluate their effect on embryo development. The results of embryo development in our study did not show differences in embryo development between treatments (Chapter 3). Because cAMP could affect different pathways, the use of NPPC+E demonstrated that the main effect of dbcAMP in the oocyte is to

delay the meiotic resumption. This conclusion was supported by the fact that the combination of NPPC+E+dbcAMP did not show any difference in embryo development compared with other meiotic resumption delay treatments. So, the new combination of substances (NPPC+E and NPPC+E+dbcAMP) can be used to delay meiotic resumption to maintain meiotic arrest.

3- Why NPPC alone did not have an inhibitory effect and why did it need the addition of estradiol to inhibit the meiotic resumption?

The NPPC treatment prevented meiotic resumption by increasing cGMP production in cumulus cells, leading to an increase in the cAMP level in oocytes [362]. However, the results of study 1 (Chapter 3) demonstrated that using 1000 nM of NPPC during the first 22 hrs of IVM did not prevent meiotic resumption. Thus, it could be hypothesized that the NPPC level used in our study was not sufficient to stimulate enough cGMP production to inhibit phosphodiesterase 3A and degraded cAMP in oocytes [339]. The cGMP production in oocytes has been positively correlated with the NPPC levels in maturation media [355]. Therefore, it was expected that the inhibition of meiotic resumption require a higher level of NPPC to produce a sufficient amount of cGMP. The NPPC acts through NPR2 receptors in cumulus cells to stimulate cGMP. However, the NPR2 expression in cumulus cells decreased during the maturation period, even with the presence of NPPC in maturation media [339, 355]. The results showed (Fig. 3.4-A) that NPPC lost its inhibitory effect on meiotic resumption and it could be related to a reduction of NPR2 expression and insufficient production of cGMP in oocytes. .

Many studies have found that estradiol can increase NPR2 expression in the cumulus cells of mouse [100], pig [132, 355] and goat [339] oocytes. Therefore, it was assume that stimulating NPR2 expression by using estradiol in the presence of NPPC could produce a sufficient amount of cGMP to maintain meiotic arrest. The combination of NPPC plus estradiol in this study (Fig. 3.4-D) efficiently prevented meiotic resumption over the first 22 hrs of IVM. Interestingly, a recent study in pig oocytes found that NPPC and estradiol in maturation media increased the cGMP level significantly compared to using estradiol or NPPC individually [100]. This finding supports the results of my thesis. In conclusion, NPPC requires estradiol to inhibit the meiotic resumption of pig oocytes.

4- Why is NO treatment not acceptable for delaying meiotic resumption?

Nitric oxide (NO) plays an important role in meiotic arrest by stimulating cGMP production in cumulus cells [450]. Based on results of study 1 (Chapter 3), the minimum level of NO required to inhibit meiotic resumption was 1mM (Fig. 3.1). When NO was used in combination with NPPC, cAMP, E, and/or progesterone to delay meiotic resumption, the inhibition process was irreversible (see Appendix -8.2). Interestingly, removing NO from this combination allowed oocytes to resume meiosis after removing the inhibitory substances. The problem with using NO may result from the inhibition of the cumulus expansion. In our study, the degree of cumulus expansion was lower than with other treatments after NO treatment (Fig. 3.2), which suggests that the gap junctions are still present and the inhibition factors can be transferred from cumulus cells to oocytes. It has been reported that NO prevents the activation of LH-induced mitogen-activated protein kinases (MAPKs) 1 and 2, that plays a role in breaking down the gap junction and stimulating cumulus expansion [117]. Although NO was able to inhibit meiotic resumption, this treatment was irreversible for meiotic resumption to put the oocytes into MII stage. In conclusion, NO is not recommended to use in delaying meiotic resumption in the concentrations that was used in our study.

5- How does delaying meiotic resumption affect oocytes–cumulus cell communications?

The main goal of delaying meiotic resumption is to maintain the communication of cumulus cells with oocytes for a longer period, in order to successfully improve the cytoplasmic maturation of oocytes. During this time, the oocytes can accumulate more molecules from cumulus cells, including proteins and RNA prior to meiotic resumption [324]. This accumulation in oocytes can support cytoplasmic maturation and improve oocyte competence [324]. Therefore, maintaining gap junction communication (GJC) between cumulus cells and oocytes is critical for enhancing the cytoplasmic maturation during meiotic arrest. The cumulus expansion reflects the nature of the communication between cumulus cells and oocytes [326]. The full cumulus expansion usually indicates a closure in the gap junctions has occurred, preventing the transport of molecules from cumulus cells to oocytes. It has been reported that GJC in cumulus-enclosed oocytes are maintained during meiotic arrest using NPPC and dbcAMP [132, 195, 235]. In our study, the degree of cumulus expansion was used to evaluate the nature of the communication between cumulus cells and oocytes. Our results showed a partial expansion of the cumulus under

different treatments (NPPC+estradiol, cAMP and its combination (CANE)) at the end of 22 hrs of IVM (Experiment 2.2). It could be hypothesized that the gap junctions between cumulus cells and oocytes during the meiotic arrest were still present. Finally, the partial cumulus expansion of COCs observed by the end of 22 hrs after delaying nuclear resumption (Chapter 3) indicated that the treatments should have maintained the communication between oocytes and cumulus cells. In conclusion, delaying meiotic resumption can maintain the GJC for a longer time based on our study; allowing oocytes to accumulate more molecules in cytoplasm that positively affect oocyte competence.

6- Why do we need to evaluate the molecular changes in matured oocytes after delaying meiotic resumption?

The changes in gene expressions can highlight many of the requirements that oocytes need to be competent. The delaying of the meiotic resumption strategy was reported to improve oocyte competence [14, 324, 451]. This strategy has been performed with many substances (cAMP, NPPC+E, cAMP+NPPC+ E) which expected to have different effects on the molecular condition of oocytes. However, no study has evaluated the molecular changes in matured oocytes after using this protocol (the two-step strategy). Evaluating the molecular pathways and the gene expression patterns can identify different biological responses that happen under certain conditions. In our study, identifying the molecular pathway and gene expression changes allows us to increase our understanding of the effects of the treatments used in oocytes during the maturation period. Understanding the behavior of these treatments at a molecular level explained the differences in our results.

7- Why do using different delaying nuclear maturation treatments cause different molecular and gene expression responses?

The environment of oocytes during IVM can critically affect oocyte competence, depending on the substances used in the IVM media [452, 453]. The maturation environment can have a major impact on gene expression in oocytes [454], which may correlate with oocyte competence [388]. The results of our gene expression study of matured oocytes (Chapter 4) indicated that using different substances in the first 22 hrs of IVM affected gene expression and pathways differently.

One possible reason for these differences in molecular responses between treatments is the various effects of substances used in the study on intracellular signal [455]. In CA treatment, the elevation of cAMP up-regulated the transcription of many genes related to the signal transduction (ARHGAOP90, RGS5, BRAF, STAT2, and STAT2) between cytoplasm and nucleus [455]. Some of these signal transduction factors play an important role in oocyte competence. The JAK/STAT signal pathway is an example of an up-regulating pathway for a transducer signal involved in cell proliferation and development that supports oocyte competence [456, 457]. In addition, increasing the level of cAMP activated protein kinase A that up-regulates genes involved in the signal from cell membrane to the nucleus (such as BRAF gene that encoding B-Ref protein) [458] and involves signals that are important for oocyte competence. The B-Ref plays an essential role in regulating the MAP kinase/ERKs signaling pathway that affects cell division, differentiation and development of a cell. The ERK pathway demonstrates a direct link between growth factor signaling to ribosome biosynthesis, via the ERK-dependent phosphorylation of BRAF [45]. These results indicated that CA treatment can support oocytes development by activation MAPK pathways and genes participating in oocyte competence (such as MRPL3 and HSP90AA1) [389, 395].

Although the NPPC+estradiol (NE) treatment has been shown to increase the level of cAMP in oocytes [100] similar to dbcAMP treatment, the gene expression and pathways did not show the same molecular response. This result may reflect the effect of different substances in each treatment (NPPC and estradiol with NE; cAMP with CA treatment) on molecular pathway changes. In this treatment, an elevation in the cGMP level in cumulus cells is expected and should transfer to oocytes [459]. The PPARG signaling pathway, which plays an important role in the metabolic process and female fertility, was found to be up-regulated under the effect of PKG (stimulated by cGMP) [460]. Many of up-regulated genes in NE treatments are involved in MAPK pathways such as: MAPK9, MAX, and REL that support oocytes development and growth [390, 398]. Also, the NE treatment activated the oxidative stress response pathway that reduce the reactive oxygen species damage in oocytes during IVM [402]. Therefore, these different responses of oocyte to NE treatments can support oocyte development and growth leading to embryo development.

It was expected that the CANE treatment would have many common genes differentially expressed compared to other treatments. However, the number of the genes in common between

CANE with CA and NE treatments was limited to single gene (Fig. 4.1). It is important to consider that we used a two-fold cut-off value in change gene expression in treated oocytes compared to the control (Pff), and this limited us to a specific number of genes. However, when we considered genes with an expression less than a two-fold change from the control, we observed more genes in common among the treatments (data not shown). The other possibility for the low number of common genes among treatments is the interaction between cAMP and estradiol functions could change profile of gene expression in oocytes during *in vitro* maturation. Studies have demonstrated this interaction, specifically, the effect of cAMP on modulated functions of the estrogen receptor [383]. Estradiol has been documented to increase the cAMP production in sufficient level to stimulate response to mediated gene transcription [461]. In an example of this interaction, a study demonstrated that the gene expression of IHCGR and ESR2A following a stimulation by cAMP and estradiol was significantly different compared to the use of estradiol or cAMP alone [387]. Therefore, it is possible that the interaction of estradiol and cAMP in CANE treatment on gene expression could induce the expression of different genes compared to CA or NE

Overall, although the treatments to delay meiotic resumption were at the same level, our results indicated that the effects of each treatment at the molecular pathway and gene expression level were different due to the different substances. However, these differences in molecular responses to various treatments did not affect embryo development among the treatments (Chapter 3).

8- Can we use a biosecure method to vitrify pig embryos?

The embryos derived from oocytes in pigs are extremely cryosensitive due to cytoplasmic *in vitro* maturation conditions and the high lipid content [462, 463]. Pig embryos were successfully vitrified using opened carrier devices, such as open pulled straw and cryoloop [423, 464]. This success was due to a rapid cooling rate, resulting from direct contact with liquid nitrogen [313]. However, the direct contact of LN_2 can increase the possibility of embryo contaminations by microbial pathogens and viral agents [430]. In our final study (Chapter 5), we evaluated the ability to vitrify embryos (morula) using a closed carrier (FS) compared to an opened carrier (OPS) in order to increase the biosecurity of the storage procedure. The results of this study showed that 62% of embryos vitrified with the closed carrier straw had a quality grade 1 after warming and culture for 48 hrs. These results indicated that the vitrification of the embryo using

both methods maintained a high rate of grade 1embryo quality. However, FS method prevented degradation of embryo quality compare to the usage of OPS after warming and 48 hrs of culture. Furthermore, the results of cell integrity (Fig. 5.1, Chapter 5) showed that almost 80% of morula had few cells stained with PI (< 4 cells), indicating good cell integrity in embryos preserved with both vitrification procedures. These different results indicated that a low cooling rate associated with closed carrier devices may not be critical to the quality of embryos vitrified using this method.

However, the vitrified embryos did not develop to further stages after warming and culturing them for 48 hrs. Suboptimal culture conditions could be a major factor affecting the yield and quality of pig embryos produced *in vitro*. The difficulty in standardizing the conditions of the culture is one of the reasons associated with variable results in embryo development using different culture media [443]. The difficulty to meet specifically all the requirements for embryo development during *in vitro* culture can be an important obstacle [191].

Assessing the integrity of cells using PI staining for embryos cannot confirm the viability of the embryo. The cryopreservation procedures can damage cells of the embryo during the vitrification or warming process. The number of dead cells in the embryo is important in determining embryo development and quality [465]. It was demonstrated that when the proportion of dead cells in a vitrified-warmed bovine embryos did not exceed 10%; and an embryo in this range of dead cells was considered to be fit for survival and development [466]. Also, it was found that an increasing the number of dead cells (stain with PI) in mouse embryos reduced the quality of embryos but did not kill the embryos [467]. In our study, PI permeability in both methods showed that >90% (Chapter 5, Fig. 5.2) of embryo had ≤ 4 cells stained with PI after vitrification, warming and culturing for 48 hrs. We can hypothesize that these embryos were in the range to fit for survival and development. However, our pig embryo did not develop into a blastocyst stage, which could be due to the culture conditions (as previously discussed).

The importance of having a biosecure method relates to the ability to store embryos in a way to prevent possible contamination. This topic is important because pig industry faces big challenges due to potential outbreak of diseases such as Porcine Epidemic Diarrhea (PED) and Porcine Reproductive and Respiratory Syndrome (PRRS) [468]. Live replacement animals may carry diseases and contaminate a seed-stock (nucleus) of a farm if the biosecurity measures are not respected adequately. The semen is another possible source for disease outbreak through

movement of semen between farms. Artificial insemination using contaminated semen in multiple sows could severely increases the risk of disease outbreaks. Therefore, semen testing is a critical procedure before shipping to prevent disease distribution [469]. If a pathogen is not detected in the semen, it could spread rapidly in multiple nucleus farms.

Thus, it is important to find a ways to reduce the possibility of direct transmission by live animals moving between different farms in order to control disease outbreaks. Using vitrified embryos under biosecured conditions, could help to reduce the possibility of contamination because these embryos can be tested before being introduced into a recipient. In addition, this method can be applied in many areas requiring protection of embryos over a long term, such as preserving embryos in a cryobank or transferring embryos nationally or internationally.

9-How different techniques that we investigated in our study can be applied in commercial work?

Many reproductive techniques have been applied in domestic animals at the commercial level to improve the productivity and product quality, such using embryo transfer, IVP, artificial insemination, and cryopreservation. IVP is one of the techniques that are used with embryo transfer to introduce new genetic material to improve the performance of herd productivity. In the pig, the IVP performance is still not efficient to produce viable embryos. Our study used a strategy to delay meiotic resumption during the first 22 hours of IVM to improve the competence of the pig oocyte to sustain embryo development after fertilization. Our results did not demonstrate an advantage to delay meiotic resumption for the *in vitro* production of pig embryos. However, we demonstrated that these embryos can be produced to an equivalent rate in a defined medium; leading to a potential commercialization of the IVP of pig embryos. Also, the vitrification of pig embryos is an important technique that can be used in different applications, such as embryo transfer or long-term conservation. Although the opened carrier has been successfully used in pig embryo verification, this method is not acceptable for the transport of vitrified embryo internationally. The International Embryo Technology Society (IETS) recommends, in term of biosecurity, using the closed carrier to transfer vitrified embryo abroad. In our study, we found FS (closed carrier) can provide equivalent results in pig embryo vitrification to OPS (opened carrier). Thus, FS can be used commercially in vitrification of the pig embryo and to transport embryos between countries with lower costs than using live animals.

6.3 FUTURE DIRECTIONS

The results of the different studies conducted in this thesis highlight some future directions for research that can help to improve IVP in pigs, thereby increasing our understanding of oocyte competence. The following points are suggestions for future studies that could be performed:

1- Based on results from chapter 3, delaying meiotic resumption had an embryo development equivalent to using the standard procedures (Pff). However, enhancing the results of our treatments may require more than just delaying meiotic resumption. Improving the maturation conditions during meiotic arrest by using additives may help to enhance oocyte competence. Many studies have indicated the possibility of improving the developmental competence of oocytes by using appropriate additives in the maturation media during IVM, such as: ascorbic acid [47], GDF9 and BMP15 [48, 49]. Therefore, it is possible to use of additives during the inhibition of the meiotic resumption period to influence embryo development positively.

2- Another possible suggestion for improving oocyte competence is to increase the period to delay meiotic resumption. The results of our study showed that using 22 hrs to delay meiotic resumption by using different treatments had the same effect on embryo development as Pff treatment. Therefore, increasing the communication time between cumulus cell and oocytes may result in the accumulation of more molecules that could have additional positive effects on cytoplasmic maturation and, consequently, oocyte competence. We can hypothesize that increasing the time of meiotic inhibition greater than 22 hrs; (mean 22-32 hrs of IVM) could have beneficial results. The reason I would selected this period is that under personal observation in our lab, we found that incubating the nuclear-delayed oocytes for 8-10 hours with maturation media with LH can oocytes reach MII (around 60%).

3- During study 1 (Chapter3), it was observed that NPPC with 1000 nM could not prevent meiotic resumption without adding estradiol (100 ng/ml) to maturation media. However, there is question: If we add estradiol to lower concentrations (100, 250, 500 nM) of NPPC that were used in this study, can they effectively prevent meiotic resumption? A dose-response was not done in this study, due to previous results obtained in our lab demonstrating the optimal concentration to be used in our strategy. This new interaction between estradiol and NPPC demands to investigate again the dose response effect of estradiol in combination with estradiol.

4- Further research needs to evaluate the effect of delaying meiotic resumption on gene expression related to oocyte competence in cumulus cells. The cumulus cells play an important role in oocyte competence [5, 50]. Many gene markers (hyaluronan synthase 2 (HAS2), inhibin bA (INHBA), epidermal growth factor receptor (EGFR), gremlin 1 (GREM1), betacellulin (BTC), CD44, tumor necrosis factor-induced protein 6 (TNFAIP6), and prostaglandin-endoperoxide synthase 2 (PTGS2)) were identified in cumulus cells related to oocyte competence and cumulus cell functioning in both human and bovine subjects [52, 53]. The effect of each inhibitory treatment on gene expression of cumulus cells could help to determine their roles on oocyte competence and cumulus cell functions.

5- Further research is needed to evaluate the effect of using embryos derived from the delaying meiotic resumption strategy (two-step strategy) in the vitrification procedures. Pig embryos are sensitive to cryopreservation procedures [463], which could suggest an insufficient cytoplasmic maturation of oocytes [38]. Delaying meiotic resumption is expected to improve cytoplasmic maturation [324]. Therefore, it is expected that the embryo derived from delaying meiotic resumption will be more competent and can be vitrified successfully.

6- The results of vitrified embryo after warming (Chapter 5) did not develop a further stage. The possible reasons for this problem were discussed earlier in chapter 5. The key to understanding this problem is to identify the viability of vitrified embryo. Staining some cells with PI in embryo can indicate a damaged embryo, but not a dead embryo. Transferring the vitrified embryos to sows may help to confirm the viability of an embryo and its ability to reach further stages of development.

6.4 WHAT IS THE OVERALL CONCLUSION OF THE THESIS?

1- Delaying meiotic resumption in pig oocytes during IVM can have a positive effect on the results of embryo development comparable to using Pff treatment.

2- Meiotic resumption in pig oocytes can be delayed successfully using the following treatments: NPPC (1000 nM) + estradiol (100 ng/ml), dbcAMP (1 mM) or the combination of both treatments for 22 hrs.

3- Different media that were used in delaying meiotic resumption strategy represent can be alternatives to using Pff during IVM. A standardization of the maturation media for IVM in pigs is required in order to make IVP more appropriate for use in commercial applications.

4- Our microarray study indicated that each inhibitory treatment has its own molecular effects that enhance the level of oocyte competence resulting in embryo development.

5- French straw carrier (a closed system) was found to be equivalent to using the OPS method (opened system) to vitrify pig embryos. From biosecurity point view, using the French straws can be the recommended method used to vitrify pig embryo, as they offer full protection from contamination during storage in liquid nitrogen.

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CHAPTER 8

APPENDIX

Introduction

This research conducted numerous trials to develop a strategy to improve IVP in pig. During our study, there were many challenges to achieving acceptable IVP results. The obstacles were typical as there are many issues that continue to need to be overcome in pig IVP. This appendix describes the different trials that were run and procedures developed during the research.

8.1 *In vitro* embryo production obstacles

In vitro embryo production during this study has many obstacles that for a time delayed our progress. Between August and October 2012, the cleavage rate and blastocyst rates ranged between 50-70% and 0-20%, respectively. However, the cleavage pattern and blastocyst rate declined over time, especially by mid-October and several months later (Fig. 8.1). Various modifications were applied to resolve this problem. In general, the cleavage rate during October 2012 to May 2013 ranged between 0-25% and there was no blastocyst production (Fig. 8.1). Over this time, many trials were run to investigate this problem and to check on the following considerations:

A- Microbial or viral contamination: Most the equipment in the lab was sterilized and samples were checked for viral contamination (negative results) in the microbiology lab. Therefore, it was assumed that microbial or viral contaminations were not the reason for this problem.

B- Nuclear maturation rate of oocytes: The rate for oocytes that reach MII is critical to achieving normal embryo development. Most the results of nuclear maturation (MII) had a rate of around 70%.

C- Ammonium concentration: In March 2012, our group was made aware of a high level of ammonium in the college. The embryos are very sensitive to ammonium and this may have been the reason for the low cleavage rate. To deal with this problem, a benchtop incubator was put into use to produce a positive air environment. As such, ambient air cannot reach the fertilized eggs. The cleavage rate increased (30%) and morulas were

produced. However, there were no blastocysts produced. To test the possibility that ammonium was the main factor affecting the production of a blastocyst, an air purifier was purchased to capture the ammonium. There was some improvement regarding the viability of oocytes and production of a few blastocysts. However, this did not reach the level obtained in August and September 2012.

D- Evaluation of semen concentrations during IVF: During the evaluation period for the IVP protocol, the concentration of semen added to oocytes during IVF was assessed. The oocytes were matured using Pff (10%). By the end of IVM, different semen concentrations (10000, 5000, 1000, 500, 250, 100 and 50 sperm/oocyte) were tested during IVF. The results for cleavage rate were variable between 0-30%, and no blastocysts were produced.

E- Using oocytes of sows for IVF: Sow oocytes are usually more competent than oocytes from pre-pubertal gilt. Therefore, sow oocytes were used for IVP with the expectation of better results associated with oocytes that are more competent. However, the cleavage rate continued to only range between 8-10% with no blastocyst production.

F- Evaluation of different culture media: Different culture media were evaluated to identify the effect of culture media on the results of IVP. NCSU-23 [150] that was prepared commercially or in the lab and E-BLAST (Minitube, Canada) were used during IVC. However, there was no improvement in cleavage rate (0-20%) and most of the cells were degraded. Recently, our lab followed a new strategy during IVC using NCSU-23. The energy recourses during the first two days of IVC used sodium pyruvate and sodium lactate which replaces glucose for the remaining seven days of the culture period. We found using this strategy helped to improve the cleavage and blastocyst rate.

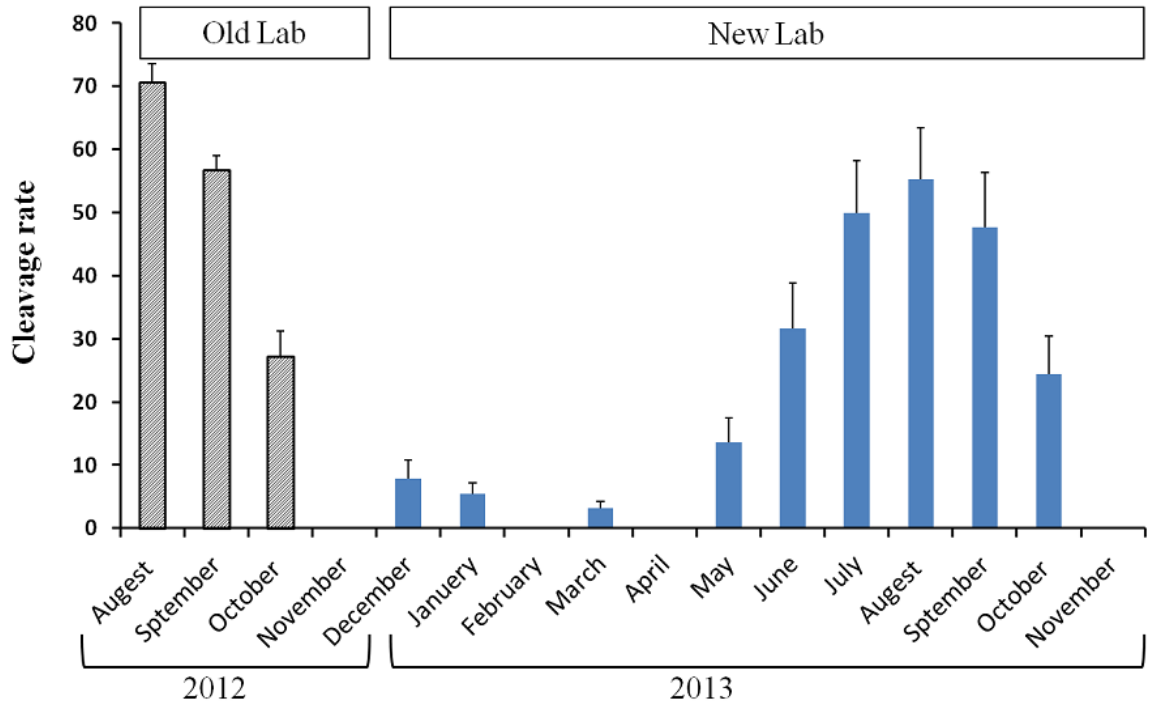

Fig. 8.1: The cleavage rate that were obtained over one year using the old lab and new laboratories.

8.2 Effect of a mix of inhibitors on final nuclear maturation

The aim of this experiment was to determine the effect of exposure of oocytes to a mixture of substances that inhibit meiotic resumption for different periods during IVM on nuclear maturation and embryo development. COCs were aspirated and selected to transfer into a maturation medium supplemented with nitric oxide (1 mM), NPPC (1000 nM), cAMP (1 mM) estradiol and progesterone (100 ng/ml). The mix of substances was added to maturation media (without LH) from the beginning of IVM for different lengths of time (2, 6, 18, 22 hr). Then, the COCs were transferred to regular maturation media (with LH) to complete the IVM period (44 hr). By the end of IVM, the COCs were denuded and the oocytes fixed in 4% formaldehyde for staining using a LAMIN/DAPI stain to assess the nuclear maturation stages (Fig. 3.1-A). The results showed that when oocyte exposure to this mixture was from 6 to 24 hrs of IVM, more than 50% of oocytes did not initiate meiotic resumption after removing the mixed media. The rate of oocytes in the GV stage increased significantly with an increase in time of delaying

meiotic resumption. However, removing NO from the mix was found to improve the rate of oocytes that reached MII after nuclear delay resumption (Fig 8.2-B). The results of this work indicated that NO with a dose of 1 mM was irreversible for meiotic resumption on oocytes. This effect increased with longer NO incubation with oocytes during IVM.

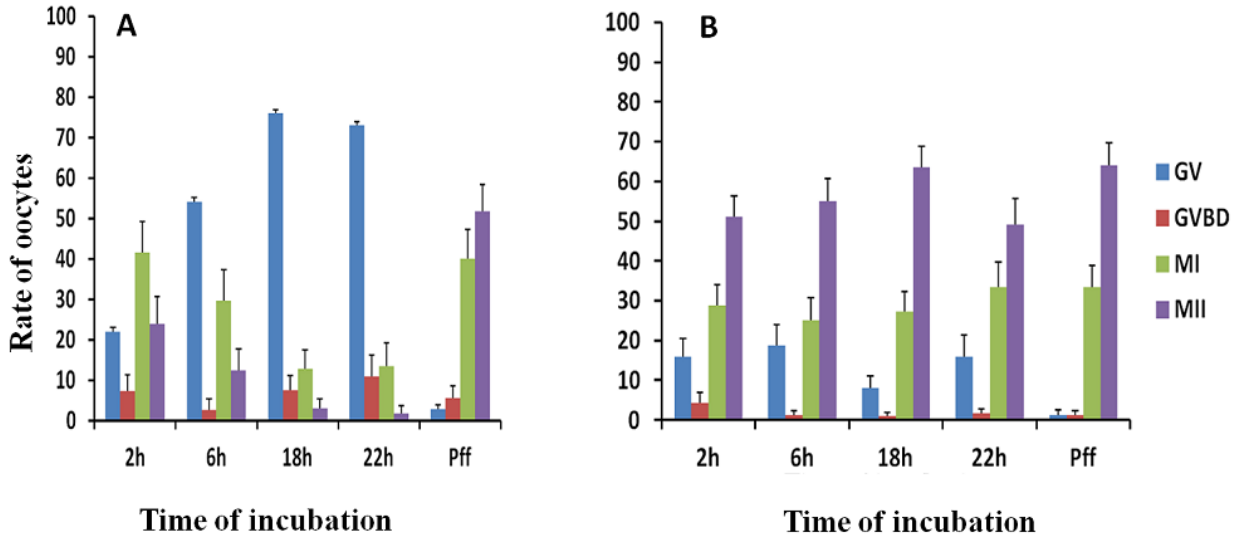

Fig. 8.2: Effect of a mixture of substances on nuclear maturation rate by the end of IVM. A) The nuclear maturation rate for oocytes treated with a mixture including cAMP (1mM), NO (1mM), NPPC (1000 nM) plus estradiol and progesterone (100 ng/ml). B) The nuclear maturation rate for oocytes treated with same mixture without NO.

8.3 Oocytes and embryo viability test

The cell viability assay used in this research is a procedure to determine whether cells are living or dead based on staining with propidium iodide (PI) that binds to DNA by intercalating between the bases with little or no sequence preference. During experiment 1 (Chapter 3) and experiment 3 (Chapter 5), the viability of the oocytes and embryos were studied for a complete assessment of the effect of treatments. The following procedure was adapted from a protocol compiled from LIVE/DEAD® Sperm Viability Kit assays (L-7011).

Procedure:

A. Prepare washing buffer (0.1% PVA) by dissolving 0.05 g of PVA in 50 ml PBS 1X.
B. Wash the denuded oocytes or embryo 3X with washing buffer.
C. Add 5 ul from the propidium (PI) solution (Invitrogen, 1 mg/ml) to the 1 ml washing buffer and mix them by pipetting.
D. Transfer the oocytes or embryos to a propidium iodide solution (from C) with a minimum aliquot from a washing buffer washing buffer (A).
E. Incubate the sample for 7 minutes at 38.5ºC.
F. Wash the oocytes with washing buffer 3X.
G. Pick out the oocytes and mount them with minimal media on slides, add cover slips (with Vaseline/paraffin on four corners) and gently press down on the cover slip or put them in a droplet of washing buffer.
H. Observe oocytes under an epifluorescence microscope.
I. All oocytes that have a red stain in the nucleus are considered dead, while others that are not stained are considered alive.

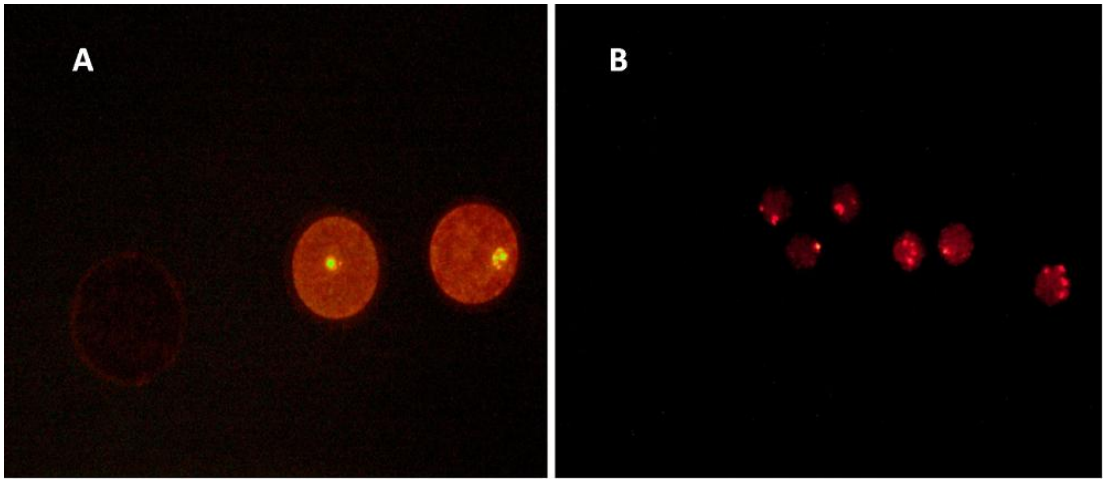

Fig. 8.3: A) Oocytes were stained using PI to identify dead cells B) Morula were stained with PI and the fluorescent spots show the number of cells that have memberanes that allow PI to penetrate.

8.4 Effect of delaying meiotic resumption using NPPC+ estradiol during IVM on embryo development of Meishan sows

This experiment evaluated the effect of using NPPC+E as a delay resumption of nuclear strategy during IVM on oocytes from Meishan sows to improve IVP performance. The COCs were transported from the USA in a maturation media with NPPC+E treatment. When the COCs were received after a 22-24 hr of shipping time, they were washed in maturation medium containing LH, but without inhibitory substances. They were then incubated for another 22 hrs.

By the end of IVM, the process was continued with IVF and IVC as described in chapter 3. The results of embryo development are described in Table 8.1. In general, the blastocyst rate was around 10%. This work still needs further evaluation because the result came from only one replicate and the effect of transport on COCs from different sources may affect embryo development results.

%(n)	Day 2	Day5	Day7	Day9
1-cell	78.6(77)	29.6(29)	20.4 (20)	16.9(11)
2-cells	5.1 (5)	12.2(12)	19.4(19)	23.1(15)
4-cells	16.3(16)	31.6(31)	16.3(16)	21.5(14)
Morula	0	15.3(15)	13.3(13)	1.5(1)
Blastocyste	0	4.1(4)	5.1(5)	6.2(4)
Dead cells	0	7.1(7)	9.8(10)	30.8(20)

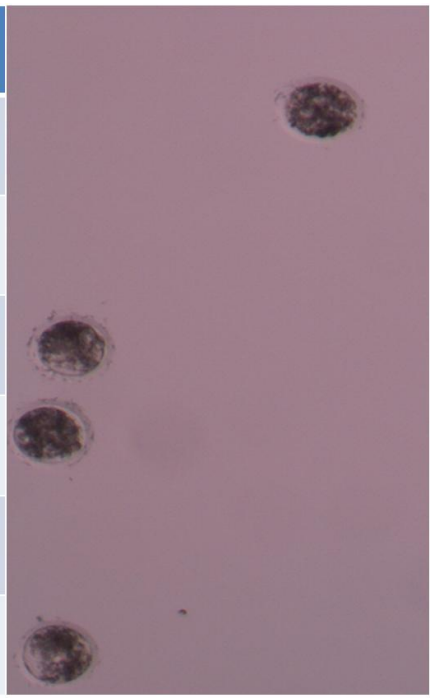

Table 8.1: The results of embryo development derived from oocytes of Meishan sows. These oocytes were treated with NPPC+E to delay meiotic resumption and transported from the USA to our laboratory. After first 22 hrs of IVM with treatment, oocytes were transferred to maturation media without inhibition substances to complete the rest of IVM period.