Effect of Pre-maturation of Immature Oocyte with a C-type Natriuretic Peptide (CNP) on Maturation and Subsequent Embryo Production from Unstimulated Mice

Christie Lee Sun
Master of Reproductive Sciences

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Department of Medicine, Nursing and Health Sciences

TABLE OF CONTENTS

Abstract

In vitro maturation (IVM) is a promising assisted reproductive technology (ART) for human infertility treatment. However, when cumulus oocyte complexes (COCs) are removed from their follicular environment when manipulated *in vitro*, it can lead to a decrease of intra-oocyte cyclic adenosine 3', 5'-monophosphare (cAMP) causing spontaneous nuclear maturation and an asynchrony with the oocytes' cytoplasmic maturation, resulting in poor embryo developmental outcomes. Nuclear and cytoplasmic synchrony is important during oocyte maturation within antral follicles.
It is maintained partially by the actions of c-type natriuretic peptide (CNP) binding with natriuretic peptide receptor 2 (NPR2), supporting high cAMP levels thus holding the oocyte in meiotic arrest. It has been reported that the addition of CNP to pre-IVM media has the capacity of maintaining cAMP levels and thus improve synchrony. Moreover, in women with advanced maternal age, successful IVM of aging oocytes faces significant challenges due to the morphological and cellular changes. Besides, there were no previous studies on new IVM strategy with aging oocytes. The primary hypothesis of this thesis was that the cAMP modulator, CNP, can inhibit the initiation of nuclear maturation during pre-IVM period and thus improve oocyte developmental competence regardless of oocyte age.

The first study used a young mouse model to investigate the exposure times (4 or 24 hours) to pre-IVM media supplemented with CNP at varying doses (0,25,50,100,150 and 200 nM) by measuring the maturation rate of germinal vesicles (GVs) placed in the medium. Subsequent fertilization of the GVs following maturation was also measured to assess developmental competency. Germinal vesicle breakdown (GVBD) was only totally suppressed when 200 nM of CNP was added to pre-IVM treatment for 4 hours. However, no differences were seen in the maturation rates following 4 hours of pre-IVM incubation. Blastocyst rate following 4 hours pre-IVM with 25 nM CNP (73.5%) was significantly higher than the IVM control group (45.3%, $P<0.05$). The second experiment used an aged mouse model to identify whether the dose of CNP required in a pre-IVM medium in aged oocytes is similar to young mice. All concentrations of CNP used in aged COCs efficiently maintained GVBD after 4 hours pre-incubation. The rate of hatching blastocysts in the 50 nM CNP (87.5%) was significantly higher compared with that of IVM control group (14.3%, $P<0.05$). These

results indicate that pre-IVM with CNP supplemented media had a dose and time-dependent effect on the maintenance of meiotic arrest regardless of oocyte age. CNP was not efficiently improving outcomes compared to the conventional IVM protocol in our laboratory.

Student Declaration

I certify that

The thesis is an original work of my research and has been composed by myself

The experimental work was done entirely by myself

Print Name: Christie Lee Sun

Date: 24th June 2019

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List of Tables

List of Figures

Abbreviations

3D	Three-dimension
ADCY	Adenylate cyclase
AMA	Advanced maternal age
ANP	Atrial natriuretic peptide
α-MEM	Alpha minimal essential medium
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine 3', 5'-monophosphare
CCs	Cumulus cells
CDK1	Cyclin-dependant kinase
eCG	Equine chorionic gonadotrophin
cGMP	Cyclic guanosine 3', 5'-monophosphcare
COCs	Cumulus enclosed oocytes
COH-IVF	Controlled ovarian hyperstimulation
CNP	C-type natriuretic peptide
E_2	Estradiol
EGF	Epidermal growth factor
EREG	Epiregulin
FSH	Follicle-stimulating hormone
GDF9	Growth differentiation factor
GnRH	Gonadotrophin-releasing hormone
GPR	G-protein coupled receptor
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
GTP	Guanosine triphosphate
hCG	Human chorionic gonadotrophin
IBMX	3-isobutyl-1-methylxanthine
IP	Intraperitoneal injection
IP_3	Inositol triphosphate

ITS	Insulin, Transferrin and sodium selenite
IVF	In vitro fertilization
IVM	In vitro maturation
LH	Luteinizing hormone
MI	Metaphase I
MII	Metaphase II
MPF	Maturation-promoting factor
mRNA	Messenger RNA
mL	Milliliter
nM	Nano mole
NPRA	Natriuretic peptide receptor
NPR2	Natriuretic peptide receptor 2
OHSS	Ovarian hyerstimulation
PCOS	Polycystic ovarian syndrome
PED3A	Phosphodiesterase
PMSG	Pregnant mare's serum gonadotropin
PS	Penicillin-Streptomycin
ROS	Reactive oxygen species
RNA	Ribonucleic acid

CHAPTER 1

Literature Review

1.1 Introduction

In vitro maturation (IVM) is a fertility treatment in which immature cumulus-oocytes complex (COCs) are extracted from antral follicles and matured *in vitro* before being fertilised (Edwards 1962). IVM of human oocytes is an attractive fertility treatment for women who otherwise have a contraindication to stimulated *in vitro* fertilisation (IVF). Accordingly, IVM has been proposed as an alternative assisted reproductive technology (ART) to significantly eliminate or reduce the risk of ovarian hyperstimulation syndrome (OHSS) in patients with polycystic ovary syndrome (PCOS) (Chian *et al.* 2013; Ellenbogen *et al.* 2014). Moreover, IVM treatments have a reduced drug cost burden and more patient-friendly with less number of injections the patient need to have in the process compared with routine IVF (Chian *et al.* 2013).

Another application of IVM is for fertility preservation in cancer patients who require potential oocyte-toxic cancer treatment (Ellenbogen *et al.* 2014). Conventional hormonal stimulation is not appropriate for many of these patients due to the tumor estrogen sensitivity in which the tumor will be exacerbated by exogenous stimulation (Chian *et al.* 2013). Additionally, there is an urgency associated with onco-fertility preservation and patient can face a very challenging decision regarding delaying cancer treatment in order to receive stimulation to preserve their fertility (Xu *et al.* 2009). IVM eliminates both the waiting time and hormonal stimulation risks for cancer patients making it an ideal treatment option for these patients who are already going through a difficult time.

Despite its proven need in the ART industry, widespread uptake of IVM by the ART industry has not occurred. This is due to the relatively limited success of IVM protocols when compared to conventional hormonal stimulation and the lack of knowledge surrounding maturation mechanisms. IVM derived oocytes have shown poorer developmental outcomes when compared to *in vivo* matured oocytes,

particularly with PCOS patients (Child *et al.* 2002). Its use and further development as a fertility treatment have been relatively limited compared with that of classical IVF following hormonal stimulation of ovaries (Child *et al.* 2002). The biggest challenge of inhibiting IVM success is the spontaneous nuclear maturation that occurs when immature germinal vesicle (GV) oocytes are removed from their follicular environment, despite no hormonal exposure (Pincus & Enzmann 1935; Edwards 1965; Donahue 1968). This spontaneous nuclear maturation is asynchronous with the oocyte's cytoplasmic maturation, which lags and negatively impacts further embryo development.

Moreover, no study has been conducted on IVM with oocytes from older animals; thus examining the efficacy of IVM using older mice could bring benefits to women with advanced maternal age. Aging oocytes are also associated with morphological and cellular changes that significantly lower the fertilization rates. Age causes a loss of mitochondrial membrane integrity in mouse oocytes as a result of increasing oxidative stress (Zhang *et al.* 2011; Lord *et al.* 2013). Similarly, during IVM culture of bovine oocytes, aging oocytes increase the concentration of reactive oxygen species (ROS), changing mitochondrial activity and ATP content (Koyama *et al.* 2014). It is very important to find technologies to regulate oocyte aging and improve fertilization efficiency to further benefit ART which further studies are required.

Modern approaches to ART and the increasing demand from patients for a safe, less invasive and cheaper treatment, that also induce less side effects has created an important need to improve IVM. However, there are many debates on the efficacy of published IVM protocols (Romero *et al*. 2016, Wei *et al*. 2017 & Zeng et al. 2013). It has been reported that a two-step IVM system with the aim of delaying nuclear maturation to maintain synchrony, improved developmental competence of cohorts of immature mouse oocytes (Romero *et al*. 2016). The two-step IVM system is comprised of a pre-IVM medium step and an IVM medium step. Pre-IVM medium contains a cAMP modulator, such as C-type natriuretic peptide (CNP) to maintain cAMP levels to inhibit spontaneous nuclear maturation. During the IVM step, the cAMP modulator is removed by washing in order to decrease the levels of cAMP and enable oocyte maturation. The aim of this review is to compare different supplements added to IVM culture media and discusses the advantages and disadvantages of each

method in animal and human models. An emphasis was placed on the strategy for increasing the efficiency of IVM and developing an optimal culture system of IVM in a mouse model.

1.2 Best candidates for IVM

IVM has been proposed as a viable treatment option for certain patient groups who are seeking ART treatment, such as women with POCS (Child *et al*. 2002), normal ovulatory with PCO (Walls *et al.* 2015), fertility preservation (Maman *et al.* 2011), poor ovarian responders (Liu *et al.* 2003), patients with an unexplained condition such as poor embryo development (Hourvitz *et al.* 2010) and rare situations such as nil mature oocytes in a stimulated cycle (Tan & Child 2002). The IVM process involves aspiration of immature oocytes from antral follicles and *in vitro* culture to promote maturation (Cha *et al.* 1991). Young women with PCOS still have a larger number of small follicles within the ovaries, which indicates the potential of pregnancy.

Polycystic ovary syndrome is the most common endocrine disorder causing ovarian dysfunction and anovulatory infertility (Franks *et al.* 2006; Goodarzi *et al.* 2011). Some PCOS patients respond well to the drugs that control ovarian hyperstimulation and IVF (Siristatidis *et al.* 2013). However, those drugs need to be injected daily and are related to a high cost burden. In addition, stimulation of ovulation in PCOS women may cause multiple pregnancies due to the development of numerous follicles as well as increasing the risk of OHSS (Siristatidis *et al.* 2013). Immature oocytes are aspirated without hormonal stimulation to avoid the related side effects in IVM treatment. Therefore, in the context of PCOS, IVM has been suggested to overcome these problems and achieved a successful pregnancy (Siristatidis *et al.* 2013; Trounson *et al.* 1994).

Although patients with advanced maternal age (AMA) are believed to be more prone to chromosomal aneuploidies (Hassold *et al.* 2007; Staessen *et al.* 2004) and the associated higher abortion rates and reduced implantation rates (Munne *et al.* 1995; Dailey *et al.* 1996; Marquez *et al.* 2000), a few studies have shown that a higher risk of aneuploidy has been found in early stage IVF embryos (Munne *et al.* 1997; Katz-Jaffe *et al.* 2005; Baart *et al.* 2007) suggesting that stimulated IVF may not be

considered the best ART treatment for advanced maternal age women. These risks have been estimated to rise from 1.9% aneuploidy embryos from 25-29 years old women to 19.1% in women aged 40 years or older (Hassold & Chiu 1985). These aneuploidies are believed to be the result of non-disjunction during the first meiotic division (Hassold & Chiu 1985; Hassold *et al.* 1987).

IVM has been successfully applied to an extensive variety of infertile women. Positive ART outcomes have been reported in onco-fertility preservation patients, AMA patients and PCOS patients, demonstrating the high potential of this technology, such as women who want to avoid the hormone stimulation because of repeated cycles without success, or may undergo radiation or chemotherapy treatment and don't have enough time to undergo hormone stimulation. Overall, advanced maternal age women are also a good target group to consider the possible benefit of IVM.

1.2.1 Current challenges in IVM

Pincus and Enzmann (1935) have first described the concept of IVM on rabbit oocytes. Edwards (1965) then performed IVM of human oocytes and found that the immature oocytes reached metaphase II (MII) *in vitro*. In 1970, Cross and Brinster applied IVM by administering gonadotropin priming of mice and successfully produced healthy offspring. Later on, *Cha et al.* (1991) conducted a study of IVM on human oocytes and reported a successful pregnancy and live birth with immature oocytes collected from the unstimulated ovaries. Since then, IVM has been widely accepted as a successful treatment for groups of infertile patients.

Although IVM has been considered a new ART technology since the first healthy baby was delivered from a PCOS patient (Trounson *et al.* 1994), clinical pregnancy rates of IVM treatment correlated with the number of oocytes retrieved have not been efficient: 38.5% (Chian *et al.* 2000), 21.5% (Child *et al.* 2002), and 22.5% (Le *et al.* 2005). Sanchez *et al.* (2015) revealed that retrieval of a large number of oocytes at once might not allow normal homogeneous development over the full cohort due to the variations of oocyte size, chromatin formation and mitochondrial formation. Age-related reduction in oocyte quality is also a factor that leads to lower the maturation

and implantation potential for aging women (Romeu *et al.* 1987; Lim & Tsakok 1997). The studies showed that women with advance maternal age often have poorer oocyte developmental competence, resulting in a significant decrease in embryonic development following IVF (Henderson & Edwards 1968; Tarin *et al.* 1998a; Sher *et al.* 2007). The reduced developmental competences are also likely to contribute to poor implantation and pregnancy rates in human IVM.

Another major hurdle is the synchronization of nuclear and cytoplasmic maturation, what is required to achieve full oocyte competency. The reason behind this problem is due to the physical removal of immature mammalian oocytes from antral follicle that results in spontaneous meiotic resumption (Pincus & Enzmann 1935; Edwards 1965; Donahue 1968). This spontaneous nuclear maturation is without hormone stimulation and is thought to be triggered by low levels of cAMP *in vitro* (Vivarelli *et al.* 1983; Törnell *et al.* 1990a), thereby significantly decrease the oocyte developmental competency.

A large number of studies have confirmed that physically aspirating COCs from their antral follicles may cause spontaneous meiotic resumption in mouse (Li *et al.* 2016; Tsuji *et al.* 2012; Zeng *et al.* 2014; Wei *et al.* 2017), bovine (Bernal-Ulloa *et al.* 2016), goat (Zhang *et al.* 2015) and pig (Appeltant *et al.* 2015) oocytes. This accumulating evidence suggests that cAMP concentration may be a factor in controlling the spontaneous nuclear maturation, the regulation of cAMP within the oocyte may be a solution to maintaining oocyte meiotic arrest in order to synchronize cytoplasmic and nuclear maturation *in vitro*, thereby improving IVM oocyte developmental competency (Zhang *et al.* 2010; Tsuji *et al.* 2012; Li *et al.* 2016; Romero *et al.* 2016).

The strategies used in attempt to improve IVM, such as combining hormonal priming, while also adjusting the IVM culture media may have themselves resulted in low efficacy of true IVM. Some studies have focused on improving IVM culture media, such as a two-step culture protocol (Zeng *et al.* 2014; Zhang *et al.* 2015; Romero *et al.* 2016), and other studies have focused on optimizing the quality of the oocytes by giving human chorionic gonadotrophin (hCG) or follicle-stimulating hormone (FSH) priming (Wynn *et al.* 1998; Mikkelsen *et al*. 1999) which has been named pseudo-

IVM. IVM treatment for women with PCOS has involved the use of hormonal priming, thus could be classed as pseudo IVM. According to Walls *et al.* (2015), IVM patients were first administered 100 to 150 IU of recombinant follicle-stimulating hormone (rFSH) for 3 to 6 days in order to stimulate antral follicles growth. In patients with PCOS, the administration of hCG has continued to be used and has also been adopted by other groups in the setting of fertility preservation for cancer patients (Creux *et al.* 2017). The study showed that pregnancy rates obtained from unstimulated ovaries were similar to those of patients who had stimulated treatment (40%) (Zhao *et al.* 2009), thus showing that hCG given *in vivo* is not necessary. Likewise, hCG in the *in vitro* system has been used for the culture of immature oocytes of women with PCOS. Ge *et al.* (2008) reported that the addition of hCG to culture medium did not improve oocyte maturation.

Despite some improvement in oocyte maturation rates and pregnancy rates, immature oocytes may be at different stages of development and thus a standardized IVM protocol could not be established. There are still many controversial areas of debate regarding hormone stimulation and the optimal IVM protocol. Data from previous studies can assist in tailoring an IVM system that promotes synchronization of nuclear and cytoplasmic maturation of oocytes. Further research would focus on improving the two-step IVM protocol in order to control resumption of mammalian oocyte meiosis. The aim of future research is to establish an IVM culture system using unprimed young mice oocytes and determine if the same system can be applied to both young and aged oocytes.

1.3 Follicular development

Folliculogenesis is the complex progression of the resting primordial follicles through primary, preantral and finally to the antral follicle whereby the oocyte within gains the ability to mature, fertilize and achieve competency. Developing follicles regularly leave the non-growing primordial follicle pool to join the growing pool, and the undergo atresia before puberty. After puberty, there is a continuous recruitment of follicles from the primordial follicle pool to form primary follicles. The oocyte contained within a primary follicle has a single layer of granulosa cells, which become cuboidal and proliferative (Eppig 2001).

The secondary follicles are now responsive to the gonadotrophins FSH and LH, and the growing oocytes are surrounded by several layers of cuboidal granulosa cells (Fortune & Eppig 1979; Cortvrindt *et al.* 1997). Oocytes are meiotically incompetent at this stage. During the early antral follicle stage, granulosa cell secretions result in the development of an antrum with follicular fluid. The transition from a preantral to an antral follicle is dependent upon the synthesis of steroid hormone gonadotrophins. Antral follicles are also known as tertiary follicles. The majority of these oocytes are meiotically competent. At this stage, appropriate gonadotrophins may sustain optimal follicular development. In addition, during this stage and with the influence of FSH, granulosa cells start differentiating and producing estrogen (Fortune & Eppig 1979; Cortvrindt *et al.* 1997).

During the pre-ovulatory stage, further proliferation of granulosa cells occurs and is comprised of cumulus cells and mural granulosa cells. Under the influence of the preovulatory gonadotrophins surge, oocytes continue to develop until they obtain competence to resume meiosis and progress to metaphase II before ovulation. During the follicular growth, granulosa cells play a crucial role in supporting oocytes to obtain developmental competence (Brower & Schultz 1982). Cumulus cells also start producing hyaluronic acid, which is deposited into the intercellular space causing the space between the cumulus cells to expand (Salustri *et al.* 1990a). In conjunction with the supporting cells, the growing oocyte also regulates the proliferation, differentiation, extracellular matrix and hormone activation of granulosa cells via gap junctions (Gilchrist *et al.* 2008).

1.4 Oocyte maturation

In most mammals, intact immature cumulus-oocyte complexes (COCs) are arrested at the prophase I stage (Hashimoto & Kishimoto 1988). These immature eggs undergo many dynamic events to ensure the maturation of oocytes, including the resumption of meiosis, germinal vesicle breakdown (GVBD) which results in chromosome condensation and polar body extrusion (Hashimoto & Kishimoto 1988). Oocyte maturation is comprised of nuclear maturation and cytoplasmic maturation. The nuclear maturation is the resumption of the first meiotic division, from prophase I to metaphase II of meiosis (Fulka *et al.* 1998). The nuclear maturation process is defined

by morphological changes within the oocyte. Ovulated oocytes are halted in metaphase II until fertilization (Thibault *et al.* 1987).

1.4.1 Nuclear maturation

The nuclear maturation process is mainly characterized by morphological chromosomal changes during meiosis, such as chromosomal segregation and alignment. Before meiotic resumption, the intact nuclear structure of the oocyte contains diffuse chromosomes known as a germinal vesicle (GV) (Eppig *et al.* 1994). During meiotic resumption, GVBD occurs, chromosomes start migrating to the pole and beginning division. The nuclear membrane vanishes. GVBD leads to chromosomes condensing and RNA synthesis ceasing (Motlik & Fulka 1976; Rodman & Bachvarova 1976).

The majority of mammalian oocytes are arrested in meiosis I in the antral follicles; the occurrence of nuclear maturation (also known as meiotic resumption) is associated with the presence of luteinizing hormone (LH) (Erickson 1966; Kruip *et al.* 1983; Hyttel *et al.* 1986). GVBD is the only morphological change that can be easily observed during oocyte meiotic resumption (Sirard & First 1988). However, physically removing the oocytes from the antral follicles can cause spontaneous meiotic resumption in the *in vitro* environment (Pincus & Enzmann 1935; Edwards 1965; Donahue 1968). The completion of the nuclear maturation process during IVM does not assure that the cytoplasmic component of the oocyte is completed (Combelles *et al.* 2002; Wang *et al.* 2009). Vivarelli *et al.* (1983) discovered that the maintenance of high levels of cAMP could be an effective means to inhibit GVBD. In addition, this nuclear maturation process is regulated by the oscillating activation of a cytoplasmic maturation-promoting factor (MPF) (Eppig *et al.*1994). High levels of MPF initially induce not only GVBD, but also sustain chromosome condensation. The levels of MPF decrease when the first polar body is extruded (Hashimoto & Kishimoto 1988; Eppig *et al.* 1994).

1.4.2 Cytoplasmic maturation

Oocyte cytoplasmic maturation refers to a series of complicated molecular changes, including the accumulation of mRNA, proteins, nutrients and other substrates, which are required for the activation of the oocyte and formation of pronuclei (Brevini-Gandolfi & Gandolfi 2001; Sirard *et al.* 2006). These molecular changes enable oocytes to acquire developmental competence in order to support fertilisation and preimplantation development (Eppig *et al.* 1994).

Some aspects of cytoplasmic maturation are associated with nuclear maturation. For example, oocytes undergo early stage of cytoplasmic activation by releasing low levels of intracellular calcium iron (Ca^{2+}) in response to microinjection of inositol triphosphate (IP_3), and when the intracellular Ca^{2+} levels reach a threshold, nuclear maturation will occur (Fujiwara *et al.* 1993). Wang *et al.* (2009) found that heat stress had a detrimental effect on the cytoplasmic competency rather than the nuclear competency of mouse oocytes, thereby decreasing blastocyst rates. The experiment of exchanged chromosomal spindles between metaphase II oocytes (Liu *et al.* 2003) and between aged and fresh oocytes (Bai *et al.* 2006) showed that the poor oocyte developmental competence was predominantly affected by cytoplasmic components. These findings indicate that activation of cytoplasmic maturation is more susceptible to environmental changes compare to nuclear maturation during oocyte development such as heat stress.

1.4.3 Resumption and arrest of meiosis

Meiotic resumption and arrest is greatly dependent on a delicate balance between the levels of factors maintaining the oocyte in arrest and the levels of factors stimulating oocyte maturation. The total number of oocytes is to be produced in a lifetime which are arrested at an early stage of the first meiotic division are present prior to birth in most newborn mammalian species (Eppig 1993; Tsafriri & Dekel 1994).

Before the LH surge, these immature oocytes obtain meiotic capability at the time of antrum construction completion (Erickson & Sorensen 1974), and this time is synchronized when the oocyte reaches a maximum level of cyclin-dependent kinase

(CDK1) and cyclin protein (Kanatsu-Shinohara *et al.* 2000). These competent oocytes are then arrested at prophase I until the preovulatory surge of LH. A large number of studies have confirmed that cAMP can be produced by the oocyte or by the granulosa cells, and is transported via gap junctions to inhibit meiotic resumption (Eppig *et al.* 2004; Norris *et al.* 2009). Spontaneous nuclear maturation is due to oocytes being removed from their follicular environment can be prevented by a cAMP modulator, such as CNP (Zhang *et al.*2010; Romero *et al.* 2016) or 3-isobutyl-1-methylxanthine (IBMX) (Appeltant *et al*. 2015) in the pre-IVM culture medium. The surge of LH binds with its receptor, which is primarily expressed by granulosa cells that enable oocytes to undergo meiotic resumption. As cumulus cells and oocytes lack LH receptors, the LH signal indirectly triggers resumption of meiosis (Peng *et al.* 1991; Eppig *et al.* 1997).

Oocyte meiotic competence is also related to other factors. Oocytes retrieved from larger follicles had better development compared to smaller follicles (Lonergan *et al.* 1994; Xiao *et al.* 2015). In addition, the size of mammalian follicles can be used as a non-invasive marker to determine the developmental ability of oocytes and improve reproductive outcomes. Specific follicle with diameter of 300-350μm had significantly higher percentage of MII and the best oocyte development to blastocysts in mice (Xiao *et al.* 2015). Other than that, maturation-promoting factor (MPF) normal activity depends on the same amount of kinase catalytic $p34^{cdc2}$ subunit protein (Gautier *et al.* 1988; Dunphy *et al.* 1988) and B-type cyclin protein present (Labbé *et al.* 1989; Gautier *et al.*1990). Pig oocytes isolated from preantral follicles less than 80μm indicated that the $p34^{cdc2}$ subunit of MPF levels is limited during the early stage of follicular growth (Hirao *et al*. 1995). A diameter of 90μm in pig oocytes indicated that $p34^{cdc2}$ catalytic subunit and B-type cyclin levels are relatively higher compared with those of fully-grown oocytes (Christmann *et al.* 1994). Furthermore, activation of MPF can also trigger meiotic resumption. The components of MPF, $p34^{cdc2}$ catalytic subunit and B-type cyclin are also present in meiotically dividing oocytes (Christmann *et al.*1994). MPF increases in the growing oocytes, and oocytes acquire meiotic competency when MPF reaches its threshold levels (Heikinheimo & Gibbons 1998).

The increasing estrogen levels trigger a significant spike in LH, causing release of the oocyte from the mature follicle and activation of oocyte maturation. The corpus luteum (ruptured follicle) starts secreting progesterone and estrogen in order to prepare the uterus for pregnancy (Vanderhyden & Tonary 1995). The levels of cAMP in the oocyte and granulosa cells are reduced just before ovulation and thereby decreasing its inhibitory impact on meiosis. Following MPF activation, the breakdown of the germinal vesicle drives the oocyte towards meiosis (Chen *et al.* 2010). After GVBD, the ovulated oocyte is halted at the metaphase II until fertilization. Sperm fusion with the oocyte causes an increase in intracellular Ca^{2+} and initiates the breakdown damage of endogenous cyclin. The onset of the chromosome segregation during the transition from metaphase to anaphase enables the oocyte to complete meiosis. At this stage, chromosome segregation errors can lead to aneuploidy, and women with advanced maternal age have increased risk of aneuploidy that can result in miscarriage later in the pregnancy (Tsutsumi *et al.* 2014).

1.5 Cyclic nucleotide and control of oocyte maturation

The cyclic nucleotides, including cAMP and cyclic guanosine monophosphate (cGMP), play a critical role in regulation of mammalian oocyte meiotic arrest and resumption *in vitro* (Li *et al.* 2016). The cAMP is an essential signaling molecule, which is synthesized from adenosine triphosphate (ATP) by active G-protein coupled receptors and membrane-bound adenylate cyclase within the cell (Horner *et al.* 2003; Hinckley *et al.* 2005). It can also be produced from cumulus cells as the result of LH or FSH stimulation, and is continuously supplied to the oocyte through gap junctions (Schultz *et al.* 1983; Racowsky 1985). Phosphodiesterases (PDE3A) is a second regulator for cAMP levels in oocytes and it is involved in the hydrolysis of the cyclic phosphate bond in cGMP and cAMP to produce 5-GMP and 5-AMP (Törnell *et al.* 1990a; Norris *et al.* 2009). The cGMP is synthesized from the natriuretic peptides pathway. Those guanylin peptides bind transmembrane guanyl cyclase, which catalyses the conversion of guanosine triphosphate (GTP) into cGMP. Much like cAMP, the cGMP acts as a second messenger to active intracellular protein kinase phosphorylation and impacts the effect of different PDE3A (Törnell *et al.* 1991).

Before the LH surge, high levels of oocyte cAMP keep the oocyte meiotically arrested. The granulosa cells also supply cGMP to the oocyte which inhibits PDE3A activity. After the LH surge, cGMP levels decrease and induce a secondary cascade of epidermal growth factor (EGF-like protein) in the granulosa cells of the follicle, showing this cascade is required for oocyte maturation (Dekel & Sherizly 1985; Norris *et al.* 2008; Vaccari *et al.* 2009). In response to LH, cAMP levels increases, and the high levels of cAMP affect the cumulus cells and interrupt communication in the COCs (Schultz *et al.* 1983). Under these conditions, the flow of cAMP to the oocyte decreases, PDE3A inhibition is relieved and meiosis is resumed (Norris *et al.* 2009) (Figure 1).

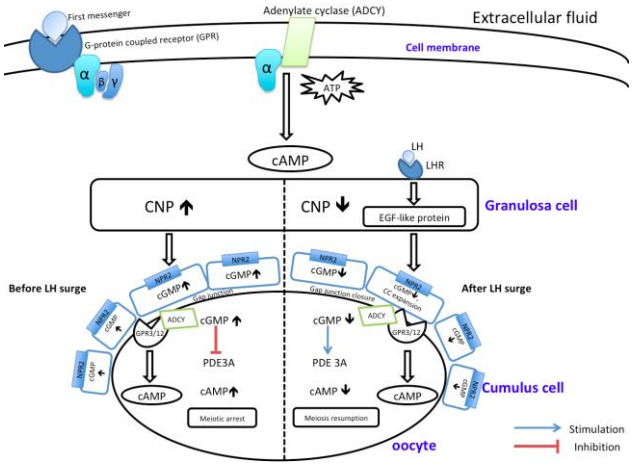

Figure 1. Proposed model depicting the role of CNP, cAMP and cGMP in maintenance of mammalian oocyte meiotic arrest before LH surge and the LH signaling pathway of mammalian oocyte resumption. First pathway of cAMP is synthesized from ATP by activation of G-protein coupled receptor (GPR) with adenylate cyclase (ADCY). It is the mainly source of cAMP supplies to the oocyte by cumulus cells via gap junction to maintain meiotic arrest. Secondary pathway of intra-oocyte cAMP is produced by the expression of G-protein coupled receptor 3 and 12 (GPR3 and GPR12) with ADCY. Before LH surge, binding of CNP with NPR2 increase the levels of cGMP, which inhibits PDE3A activity, thus maintain cAMP levels within oocyte. High levels of cAMP sustain meiotic arrest. After LH surge, the activation of EGF-like protein pathway interrupts the expression of CNP with NPR2, the levels of cAMP decrease enabling oocyte resumed.

It has been identified that meiotic arrest of oocytes *in vitro* is dependent on the preservation of high levels of cAMP within oocytes (Conti *et al.* 2002; Nogueira *et al.* 2003). The cAMP is maintained within oocyte through two mechanisms. Firstly, oocytes can autonomously produce cAMP, the expression of G-protein coupled receptor 3 and 12 (GPR3 and GPR12) is responsible for the regulation of cAMP

levels in the oocyte (Hinckley *et al.* 2005; Mehlmann 2005; Vaccari *et al.* 2008). Secondly, FSH or LH triggers the production of cAMP in the cumulus cells and flows to the oocyte through gap junction (Schultz *et al.* 1983; Racowsky 1985). This is the main source of cAMP supply to the oocyte to maintain meiotic arrest (Figure 1).

The cAMP and cGMP production is self-regulated. Törnell *et al.* (1990a) investigated the function of cGMP during rat oocyte maturation, and they reported that cyclic GMP is an effective inhibitor of cAMP degradation; thereby regulation of PDE3A and low levels of cGMP would contribute to spontaneous meiotic resumption *in vitro*. In addition, Norris *et al.* (2009) showed that cGMP enzyme is generated in cumulus cells, and it permeates from the cumulus cells into the oocyte to inhibit cAMP hydrolysis by PDE3A through gap junctions. Taken together, these promising results provide confirmation that the concentration of cAMP must be maintained at optimum levels to assure oocyte meiotic arrest.

1.5.1 CNP/NPR2 signaling in the maintenance of oocyte meiosis

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP; also known as natriuretic peptide precursor C) are the most important members in the natriuretic peptides family in most mammalian species. ANP and BNP are primarily generated in atrial and ventricular cardiomyocytes, respectively (Kangawa & Matsuo 1984; Saito *et al.* 1989). Both ANP and BNP natriuretic activities depend on their receptor natriuretic peptide receptor (NPRA or NPR1). CNP has limited activity and only binds its specific receptor. In addition, CNP-22 is the version of CNP that is identical in humans and rats (Potter *et al.* 2006). Using rat as a model may benefit human oocytes meiotic development in the further study.

The CNP is produced by mural granulosa cells, and its cognate receptors are located in cumulus cells; natriuretic peptide receptor 2 (NPR2; also known as the guanylyl cyclase B receptor) (Potter *et al.* 2006; Zhang *et al.* 2010). The gene expression of both CNP and NPR2 has not only been found in the mouse ovarian follicles from early stages of folliculogenesis (Jankowski *et al.* 1997; Gutkowska *et al.* 1999), but

also plays a crucial role during the transition of preantral follicle to antral follicle development (Sato *et al.* 2012).

There are several studies that have demonstrated that CNP, a cAMP regulator, binds with its receptor NPR2 in antral follicles to maintain oocyte meiotic arrest through the regulation of cGMP and cAMP (Tsuji *et al.* 2012; Zhang *et al.* 2010). High concentration of oocyte cAMP is crucial to maintain mammalian oocytes in meiotic arrest. Blocking of oocyte PDE3A activity is critical for supporting higher levels of cAMP (Zhang *et al.* 2010). It has been shown that there are two pathways that maintain meiotic arrest. The first is that oocytes themselves participate in the meiosis-arresting pathway by producing cAMP. The second is that the follicular granulosa cells produce CNP and act via NPR2 expressed by cumulus cells (CCs) to stimulate cGMP intracellular concentrations in order to block oocyte PDE3A activity in mural and cumulus cells and maintain meiotic arrest (Zhang *et al.*2010) (Figure 2).

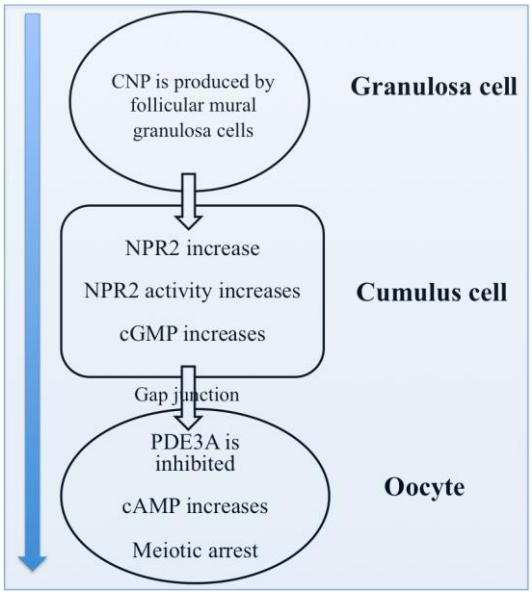

Figure 2. The production of CNP in guanulosa cells and the coordination between CNP and cyclic nuclerotides (cAMP and cGMP) in the maintenance of meiotic arrest in mammalian oocyte. Detail in text.

1.5.2 IVM systems with a cAMP/cGMP modulator

One of ART treatments for infertile patients and livestock breeding is using a standard IVM system termed spontaneous oocyte maturation. COCs are isolated from antral

follicles and cultured for 24 to 48 hours in IVM medium, which often contains a commercial cell culture medium as a base such as minimum essential medium (MEM) or TCM199 (Medium 199 Sigma Aldrich, Australia), and is supplemented with macromolecules such as albumin or serum, and hormones such as FSH (Sutton *et al.* 2003). This approach is constructed on the theory that spontaneous meiotic resumption of oocytes after removal from their follicular environment will occur (Pincus & Enzmann 1935; Edwards 1965) (Figure 3). Therefore, oocyte spontaneous meiotic resumption occurs without a cascade of cAMP and cGMP signaling (Norris *et al.* 2009; Vaccari *et al.* 2009), possibly resulting in asynchrony of nuclear and cytoplasmic maturation and reduced oocyte competence.

Based on the knowledge of the cyclic nucleotides that are involved in oocyte meiotic arrest and resumption, and the physiology of how the oocyte interacts with cumulus cells to regulate the levels of cAMP, a new approach of a two-step IVM system, has been introduced. The two-step IVM system composes of a pre-IVM step and an IVM step. Pre-IVM uses a relatively high level of PDE3A inhibitor, such as CNP or IBMX, to inhibit spontaneous oocyte meiotic resumption upon aspiration of COCs from the antral follicles, thereby maintaining cAMP concentration stimulated by FSH. In the IVM culture phase, the PDE3A inhibitor medium is washed off in order to decrease the levels of cAMP and enable oocyte maturation (Zhang *et al.* 2010; Romero *et al.* 2016) (Figure 3).

IVM studies using cAMP/cGMP modulators for oocyte meiotic control have been investigated in different mammalian species including mice (Albuz *et al.* 2010; Romero *et al.* 2016; Tsuji *et al.* 2012; Zeng *et al.* 2014), bovine (Albuz *et al.* 2010; Li *et al.*2016), porcine (Appeltant *et al.* 2015) and goat (Zhang *et al.* 2015) models. With the strategy of a two-step IVM system with different cAMP modulators to maintain meiotic arrest in mouse oocytes, the cAMP modulator Forskolin and FSH was used during pre-IVM for 1hour, followed by conventional IVM medium without FSH (Zeng *et al.* 2014). The combination of this two-step oocyte maturation medium effectively controlled meiotic arrest in pre-IVM phase and reduced spindle abnormalities compared with the control IVM group. This approach also significantly improved blastocyst rate and quality (Zeng *et al.* 2014)). Similarly, using the CNP during pre-IVM phase for 4 hours in goat COCs, Zhang *et al.* (2015) confirmed that

CNP could inhibit meiotic resumption and provide sufficient time for the cumulus cells through gap junction to synchronize nuclear and cytoplasmic maturation. Their results showed that CNP treatment groups had improved blastocyst rates and total cell number in the blastocysts.

Romero *et al.* (2016) reported for the first time the use of pre-IVM medium to improve developmental competence of intact COCs that were aspirated from unprimed juvenile mice. COCs were placed in pre-IVM culture under CNP medium supplementation containing FSH, GDF9 and estradiol for 48 hours, followed by culture in IVM culture medium supplemented with FSH. This prolonged prematuration culture system with CNP supplementation significantly improved the formation of blastocysts, indicating that pre-IVM medium containing CNP controls oocyte meiotic arrest and prolonged the time for oocyte maturation communicating with CCs through gap junctions. It was also observed that mice COCs were pre-incubated with CNP for 48 hours, which is considered the maximum pre-IVM time. Previous studies utilised times of 12 or 24 hours (Downs *et al.*1986), 1 or 2 hours (Albuz *et al.* 2010), 1 hour (Zeng *et al.* 2014) and 24 hours (Wei *et al.* 2017). The timing of meiotic resumption differs among species. In both two-step and one step IVM, the progression of GV to GVBD as induced by hormonal stimulation takes approximately 1 to 3 hours in mice (Conti & Franciosi 2018) and 8 hours in a bovine model (Sirard *et al*. 1989). Spontaneous human oocyte maturation occurs after 22 hours of *in vitro* culture (Escrich *et al.* 2012).

The cAMP modulation has been shown to be a pivotal factor in oocyte meiotic arrest. However, Appeltant *et al*. (2015) collected COCs from pigs to investigate the effect of the cAMP-modulating agent 3-isbutyl-1-methylxanthine (IBMX) during IVM. Their results showed that immature COCs cultured in IVM medium with IBMX supplementation were unable to maintain meiotic arrest in oocytes. Although several attempts have been made to improve the efficiency of a two-step cAMP modulator IVM protocol in many mammalian species, there is a lack of consistent results and sufficient information to fully validate the culture components and their concentration in the system (Appeltant et al.2015; Albuz et al. 2010; Li *et al.*2016; Romero *et al.* 2016; Zhang *et al.*2015). Subsequently, the oocyte maturation rate in a cAMP

modulated IVM system is still low in human IVM (~50%) (Shu *et al*. 2008). Thus further studies to optimize the protocol and culture conditions are needed.

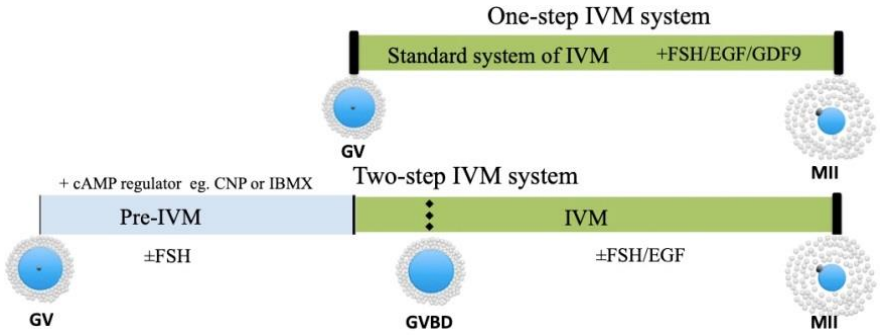

Figure 3. Different approaches of IVM system. One –step IVM system (spontaneous oocyte maturation) differs from two –step IVM system, which composes of pre-IVM and IVM phase. Intact COCs are incubated with pre-IVM media containing cAMP regulator (eg. IBMX or CNP) to maintain the levels of cAMP within oocytes for an extended period. In the IVM phase, cAMP regulator is washed out, the levels of cAMP decease enabling oocyte maturation (modified from Albuz *et al*. (2010)).

1.6 Oocytes from Aged mothers have reduced fertilization rate and embryo development

Women's fertility declines with advancing age from the mean age of 30 years-old and declines more significantly when ≥ 35 years-old which results in the progressive decreased oocyte number and poor oocyte quality (Liu, 2011). Lower pregnancy rate was found in women with advanced maternal age largely as a result of the higher aneuploidy rate compared to younger women (Grande *et al.* 2012). The effect of aging also causes complex cellular and molecular changes in oocytes (Takahashi *et al.* 2009); the changes of which may affect normal embryo development and result in early pregnancy loss in humans (Wilcox *et al.* 1998). Recent studies on murine oocytes have shown that the aging of oocytes can cause a loss of mitochondrial membrane integrity as a result of increasing oxidative stress (Lord *et al.* 2013, Zhang *et al.* 2011). The study has identified that oxidative stress acts as a key mediator, which regulates the intrinsic apoptotic pathway in the aged oocytes, thus, the oxidative stress raised as oocyte age increased result in negative effect on oocyte development (Lord *et al.*2013*).*

Chromosomal segregation errors can occur during both mitotic and meiotic divisions. During the second meiotic cell division, chromosomal segregation errors can cause DNA damage and incorrect chromosome number. Aneuploidy is a direct consequence of chromosome segregation error in meiosis. As a result, aneuploidy of fertilized eggs and embryos can cause miscarriage or with trisomy such as Down syndrome (Tsutsumi *et al.* 2014). This result was consistent with the study by Loane's team that the risk of children born with Down syndrome increased in women at advanced maternal age (Loane *et al.* 2013). The risk of women conceiving a child with DS is 1 in 1400 births at age 24, 1 in 350 at age 35 and increases to 1 in 25 by age 45 (Yoon *et al.* 1996). Moreover, the two core subunits of meiotic cohesion complex SMC1B and REC8 have a critical role to ensure precise distribution of chromosomes during meiosis. In Yoon *et al.* (1996)'s study, they showed that the decrease in cohesion concentration is associated with age-related meiotic segregation errors in mammalian oocytes. Compared with a 20-year-old, the levels of SMC1B and REC8 are significantly decreased in women who are 40 years old (Tsutsumi *et al.* 2014).

1.6.1 Detrimental effects of oocytes from women with advanced maternal age in an IVM system

The aging of oocytes is a key factor that compromises the overall quality of oocytes during IVM culture, which is associated with a decline in fertilization and embryonic development. Increased ROS production in aging oocytes subsequently causes the release of cytochrome *c*, which is involved in a key apoptotic pathway (Bobba *et al.* 1999, Tiwari *et al.* 2002).

The reactive oxygen species (ROS) can diffuse through cell membranes and significantly damage biological molecular structures, including nucleic acids, lipids and protein, resulting in dysfunctional mitochondria (Kowaltowski & Vervesi 1999) and apoptosis (Hashimoto *et al.* 2000). Excessive levels of ROS are associated with meiotic arrest in human oocytes and cell death in mouse embryos (Tripathi *et al.* 2009). In addition, an increased concentration of glucose increases the generation of ROS during oocyte maturation. When the levels of ROS exceed normal physiological conditions, the resultant oxidative stress can reduce oocyte quality in the mouse (Hashimoto *et al.* 2000; Xie *et al.* 2016).

Prolonged maturation culture could reduce the quality of mammalian oocytes and induce ageing of the oocytes during the MII arrest period (Kikuchi *et al.* 2000; Agung *et al.* 2006). The study has found that the percentage of mature bovine oocytes reached the highest level after being cultured for 22 hours, compared with oocytes cultured for 28 or 34 hours. The aged oocytes were more likely to undergo germinal vesicle breakdown (GVBD) from germinal vesicle (GV) stage during the maturation culture period (Agung *et al.* 2006). Faster developing immature oocytes showed an increased activation, a higher spontaneous fragmentation rate and a decrease in maturation promoting factor (histone H1 kinase) activity (Kikuchi *et al.* 1995; Kikuchi *et al.* 2000). Moreover, the activity of histoneH1 kinase is related to the competency of the cytoplasm of the oocyte (Kikuchi *et al*.1995). It can be seen that cytoplasmic changes affect oocyte quality when the meiotic arrest period is prolonged. Additionally, one study has shown that *in vitro* aged oocytes lost the microfilament-rich area over the meiotic spindle first, then disrupted spindle location and subsequently misplaced chromatin organization. These abnormal morphological changes of *in vitro* aged oocytes are similar with that occurring with in vivo ageing (Webb *et al.* 1986).

The development of a three-dimensional (3D) matrix has provided a valuable *in vitro* model to investigate the regulation of folliculogenesis in prepubertal, young and older adult monkey (Xu *et al.* 2010). In this study, compared with follicles from older adult monkeys, there was a larger percentage of secondary follicle survival from prepubertal and young monkey during the culture period. The lower surviving follicle rate obtained from older monkeys might be due to the preantral follicles dying as a result of lack of apoptosis inhibiting factor (Orisaka *et al.* 2006). Alternatively, the activation of other factors responsible for follicle atresia during early follicular development (Thompson *et al.* 2004) increased as a result of the aging process.

Taken together, the maturation culture period is important for oocyte maturation and subsequent successful fertilization. The mechanisms of prolonged meiotic arrest resulting in poor survival rate of follicles may be relevant to the declining reproductive ability of older women. These features of ovarian aging, including poor oocyte quality or decreased survival rate are frequently observed clinically in patients with advanced maternal age (Xu *et al.* 2010).

1.7 Conclusions and new perspectives

It is widely accepted that high levels of cAMP within mammalian oocytes during IVM could sustain oocyte meiotic arrest and substantially synchronize nuclear and cytoplasmic maturation, thus improve oocyte developmental competence. The communication between oocyte and the surrounding cumulus cells via gap junctions diffuses the levels of cAMP and cGMP in supporting oocyte meiotic arrest and resumption. The cAMP levels are produced from the ATP by the activation of GPR with ADCY, which is continually supplied to the oocyte by cumulus cells via gap junctions. A large number of studies have suggested that pre-IVM step medium supplemented with cAMP modulator, such as CNP, acts as the main defense factor against 'spontaneous' maturation, which can regulate the period of nuclear maturation. One of the cAMP modulators, CNP, binding with its cumulus cell receptor NPR2, effectively regulates the levels of cGMP in order to maintain ideal concentrations of cAMP within oocytes. A relative high level of cAMP extends the duration of nuclear maturation, which improves the synchronization of cytoplasmic and nuclear maturation and contributes to further sustained acquisition of developmental competence of oocytes. The duration of nuclear maturation plays a critical role in supporting oocyte maturation and is a crucial factor that can enhance IVM efficiency.

A two-step IVM system with different cAMP modulators to regulate levels of cAMP during pre-IVM phase is needed. However, enhancement of IVM culture conditions is complicated as it depends not only on the use of different cAMP modulators, but also many other factors. Firstly, cAMP modulators can differently influence the oocytes according to developmental stage in different mammalian species. For instance, IBMX may effectively inhibit some mammalian oocytes resumption, however, it is not able to maintain pig oocyte meiotic arrest during the pre-IVM phase. The duration of the pre-IVM phase and the concentrations of cAMP modulator also need to be considered. Secondly, even though a large number of studies have concluded that two-step IVM system improve oocyte developmental competency, these COCs were retrieved from hormone-stimulated ovaries.

In current IVM protocols, serum, hormone and growth factors have been identified as the key factors, however, synchronization of nuclear and cytoplasmic maturation during IVM is also important for the proportion of oocytes matured to MII and further oocyte developmental competence. Regulation of cAMP during the pre-IVM phase may be a solution to the current discrepancies in the literature and may promote oocytes to acquire developmental competency, with particular attention paid to the effect on oocytes from older mothers.

CHAPTER 2

General Materials and Methods of the Experiment

2.1 General materials & methods

Animal model and ethics statement

The mice used for the experiment were the C57Bl6/CBA F1 strain, aged between 5-6 weeks and 36 weeks old, respectively. All mice were delivered at 4 weeks old. The 5-6 weeks old mice were used immediately after one-week acclimatization in animal house at Monash Medical Centre; the 36 weeks old mice were kept for 32 weeks until they reached 36 weeks. F1 mice were housed in a temperature at 24°C and light controlled environment on a 12 hours light and 12 hours dark cycle. The experimental protocol was reviewed and approved by the Monash Animal Ethics Office (Animal Ethics Committee (AEC) approval number: 2017/18). The animal handling procedures followed animal ethics guidelines with the consent of the ethical committee of Monash University.

Female mice (5-6 weeks old/36 weeks old) were killed by cervical dislocation, compacted COCs were collected from ovaries and either matured directly in IVM media for 18 hours (one-step IVM system; also called IVM control group) or placed in a pre-IVM media supplemented with different concentrations of CNP for 4 or 24 hours plus 18 hours of IVM culture (two-step IVM system; also called treatment groups) all at 37°C in humidified 5% CO_2 in air. For the IVF group, female mice (5-6 weeks old) were intraperitoneally injected using PMSG (5IU/animal) and 48 hours later followed by human chorionic gonadotropin (hCG) (5IU/animal). Compact COCs were collected from the oviduct after 13 to 15 hours post-hCG injection.

Material and formulations for the Pre-IVM and IVM media

The composition of media for COCs pre-IVM and IVM is shown in Figure 4 The base medium for Pre-IVM and IVM preparations consisted of α-minimum essential medium (α-MEM; Life Technologies. Victoria, Australia) supplemented with 2.5%

v/v fetal bovine serum (FBS) (Life Technology. Victoria, Australia), 5ng/ml insulin, 5µg/ml apo-transferrin and 5ng/ml sodium selenite (ITS) (Sigma. Victoria, Australia). For pre-IVM, the base medium was supplemented with 25 nM CNP-22 (also known as CNP) (Phoenix Europe. California, USA), 10 nM 17-β-estradiol (E2) (Sigma. Victoria, Australia), 50ng/ml growth and differentiation factor 9 (GDF9) (R&D Systems Europe. Minnesota, USA) and 2.5mIU/ml pregnant mare's serum gonadotropin (PMSG) (Folligon; MSD-animal-health; Australia). Supplementations to the IVM media included 4ng/ml recombinant epidermal growth factor (r-EGF) (Roche. Minnesota, USA) and 100ng/ml recombinant mouse epiregulin (EREG) (R&D Systems Europe). The media were prepared no longer than 18 hours before use.

Collection media:	L-15	
	FBS	10% (v/v)
	PS	100IU/ml
	IBMX	200 μM
IVM basal media:	α-MEM	
	FBS	2.5% (v/v)
	ITS	5ng/ml
Pre-IVM media:	IVM basal media	
	CNP	
	E_2	10nM
	GDF9	50ng/ml
	PMSG	2.5 mIU/ml
IVM media:	IVM basal media	
	r-EGF	4ng/ml
	EREG	100ng/ml
	PMSG	2.5mIU/ml

Figure 4. The composition of media for COCs collection, pre-IVM treatment and IVM culture. Adapted from the formulation of the media as described by Romero *et al.* (2016)' paper

Collection of immature COCs from unstimulated mice

Compacted immature COCs were collected from small antral follicles of mice (5-6 weeks old) without prior gonadotropin administration. Female mice oviducts were dissected and placed in the collection media (modified Leibovitz L-15 media). Collection medium consisted of Leibovitz L-15 containing 10% v/v fetal bovine serum (FBS) (Life Technology), 100IU/ml penicillin, 100µg/ml Streptomycin (PS) (Sigma), and supplemented with 200µM 3-isobutyl-1-methylxanthine (IBMX) (Sigma) NB: IBMX acts similarly to a cAMP modulator, and is added to the media to prevent meiosis reinitiating during the period of collection (Romero et al. 2016). The ovaries were separated from the oviduct in a petri dish, using 19 and 25-guage needles

to release the immature oocytes under a dissecting microscope, and then COCs were placed in the modified L-15 collection medium. Only COCs with more than two layers of unexpanded cumulus cells were selected for maturation.

Collection of COCs from oviduct of stimulated mice

For the *in vivo* control (IVF) group, female mice (5-6 weeks old) were intraperitoneally injected using PMSG (5IU/animal) and 48 hours later followed by human chorionic gonadotropin (hCG) (5IU/animal). Compact COCs were collected from the oviduct after 13 to 15 hours post-hCG injection.

Sperm preparation

Spermatozoa were retrieved from the cauda epididymis from adult male mice between 8 to 10 weeks old (C57Bl6/CBA F1). The epididymides were placed in 1ml pre-equilibrated fertilization media (Research Fertilization media, Cook Medical, Bloomington, USA) then incubated for 30 minutes at 37°C with a loose lid in an atmosphere of 5% CO2 in air. Spermatozoa were prepared by standard swim-up. After the incubating period, the top layer was removed, mixed well and a sample (10μL) removed for counting on a Makler Counting Chamber.

2.2 Primary aim and hypothesis

The aim of this research was to examine whether the cAMP/cGMP modulator, CNP, can inhibit the initiation of nuclear maturation during the pre-IVM period (as measured by GVBD) and thus improve final maturation, fertilization and developmental competence of resultant embryos in oocytes collected from younger and older adult mice.

It is hypothesized that exposing COCs to CNP prior to traditional IVM will prevent GVBD and as a result to enhance maturation, fertilization, blastocyst and hatching rates of resultant embryos regardless of oocyte age.

CHAPTER 3

Preliminary Data: Effect of Pre-IVM with CNP on (a) meiotic arrest and maturation and (b) developmental capacity of oocytes following fertilization on young mice age between 5-6 weeks

3.1 Introduction

In vitro maturation (IVM) is a promising assisted reproductive technology (ART) for the treatment of human infertility. In recent years, social and cultural trends have led to women delaying childbirth thus results in age-related poor oocytes quality, low oocyte number and infertility (Child *et al.* 2002). Meanwhile, the average age of women seeking ART is increasing (Grande *et al.* 2012). Moreover, IVM has shown poor developmental outcomes in humans compared to those achieved with conventional controlled ovarian hyperstimulation treatment (Romero et al. 2016). A major hurdle is that, once immature oocytes are removed from their follicular environment the concentration of cyclic adenosine 3', 5'-monophosphare (cAMP) decreases, which can lead to the asynchronization of nuclear and cytoplasmic maturation. Utilizing the pathways to increase cAMP levels within the oocyte has therefore been proposed as an approach to enhance the synchronization of cytoplasmic and nuclear maturation. C-type natriuretic peptide (CNP) with its receptor 2 (NPR2) plays a paramount role in maintaining oocyte meiotic arrest in antral follicles. The natriuretic peptides are the key elements in maintaining meiotic arrest of COCs *in vivo* and thus their pathways and mode of action have been investigated for *in vitro* use. The addition of CNP to a pre-IVM media has been reported to maintain cAMP levels and thus improve synchrony (Romero et al. 2016). The aim of the experiment was to mimic Romero et al. (2016)' study to investigate the exposure times to pre-IVM media supplemented with CNP in respect to maturation rates of germinal vesicles (GVs) derived from compacted cumulus-oocytes complexes (COCs) and examine the subsequent fertilization and development of GVs following a pre-maturation period with CNP. It is hypothesized that a period of prematuration with CNP will enable nuclear and cytoplasmic synchronization and improves oocyte developmental competence.

3.2 Materials and Methods

The materials and methods were used in the experiment as described in Chapter 2.

3.3 Experimental design

The experimental design is shown in Figure 5. The total number of 249 COCs were collected from small antral follicles of 15 unstimulated female mice aged 5-6 weeks and matured either directly in IVM media for 18 hours (IVM control group) or incubated with 25nM CNP for 1 hour, 2 hours, 4 hours, 24 hours prior to IVM (Figure 5) all at 37°C in humidified 5% CO2 in air. At the end of the pre-incubation period, cohorts of COCs (approximate one third of total number) were denuded of cumulus cells using a fine glass pipette and assessed for GVBD. The oocytes nuclear status was assessed as described in Figure 6. The cumulus cells surrounding the oocyte were also assessed for signs of expansion as this gives an indication that maturation of the oocyte has been initiated. The remaining compacted COCs were transferred to IVM media for 18 hours. Following IVM, those with an extruding polar body were subjected to IVF. Matured oocytes were incubated at 37°C, 5% CO2 and saturated humidity with capacitated sperm obtained from C57Bl6/CBA male (concentration of 0.25 million motile sperm/ml. After 3 hours, presumptive fertilized oocytes were washed twice and were transferred to 30µl Cook cleavage medium (Cook Medical) with mineral oil overlay in groups of approximately 10 zygotes at 37°C, 5% CO2 in air and saturated humidity. At 20 hours post insemination, the embryos were assessed for cleavage (2 cells) and this was used as a fertilisation measure. All cleaved embryos were transferred to blastocyst media (Cook Medical) 48 hours later, and observed for blastocyst formation at 72 and 96 hours post insemination. As an *in vivo* control group, COCs were obtained from females aged 5-6 weeks old with an intraperitoneal injection (IP) of 5IU PMSG followed by IP injection of 5IU hCG 48 hours later. The mature COCs were collected from the oviduct 13 hours post hCG and inseminated with the same sperm sample and zygotes were cultured under the same conditions as the IVM oocytes.

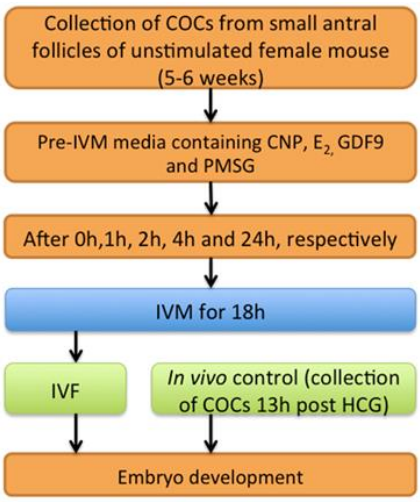

Figure 5. Experimental design. COCs were either matured directly in IVM media for 18h (IVM control group) or incubated with 25 nM CNP for 1, 2, 4, and 24 hours (treatment groups). At the end of the pre-incubation period the status of the GV was assessed. At the end of the IVM culture period the maturation status of the oocyte was assessed.

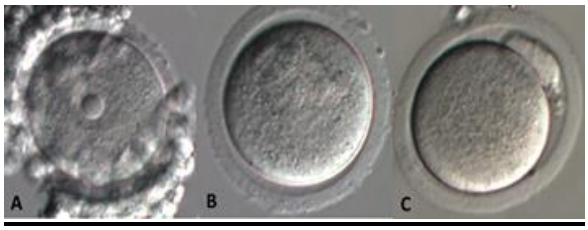

Figure 6. The Visualization of (A) Germinal vesicle (GV) was used as the criterion of meiotic arrest (B) Germinal vesicles breakdown (GVBD, also known as MI) was used as the criterion of meiotic resumption (C) MII: first polar body (PBI) extrusion was used as the criterion of completed nuclear maturation

Statistics analysis

Fisher's exact or Chi-square test of independence using the GraphPad Prism 5 software program, version 5.0c (GraphPad Software Inc., San Diego, CA, USA) were used to compare GVBD, maturation status, 2-cell, blastocyst and hatching rates between treatments. The Chi-square test with Yates' correction for continuity was used to compare treatments for numbers of 5 and less, while Fisher's exact test was used for numbers greater than 5. The level of statistical significance was defined as $P < 0.05$.

3.4 Results

Denuding post pre-IVM for each treatment time showed that after 1 hour pre-IVM incubation, CCs were strongly attaching to the oocyte, in contrast, with 24 hours pre-IVM, where the CCs became loosely attached or absent (Figure 7). GVBD was only totally suppressed in the 1 hour Pre-IVM treatment (0%) with longer Pre-IVM times causing breakdown of the GV in increasing numbers (2 hours, 17.8%; 4 hours, 20%, respectively), with up to 50% for 24 hours Pre-IVM period and of those 30% had progressed to the MII stage (Figure. 8).

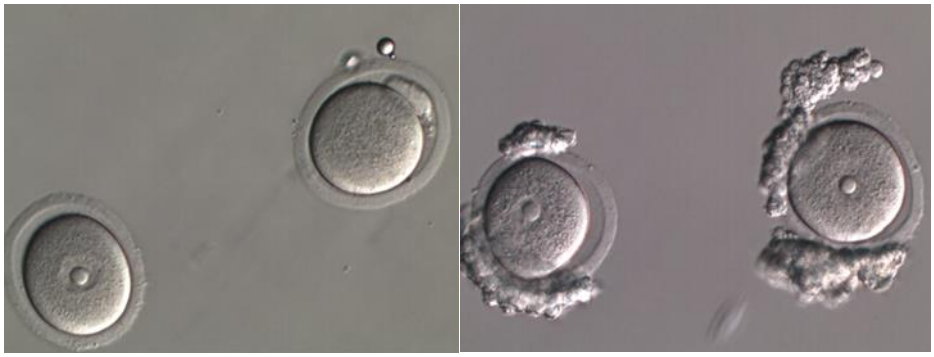

Figure 7. Germinal vesicle (GV)/Metaphase I (MI) after pre-IVM supplemented with 25 nM CNP for 24 hours (left) and 1 hour (right)

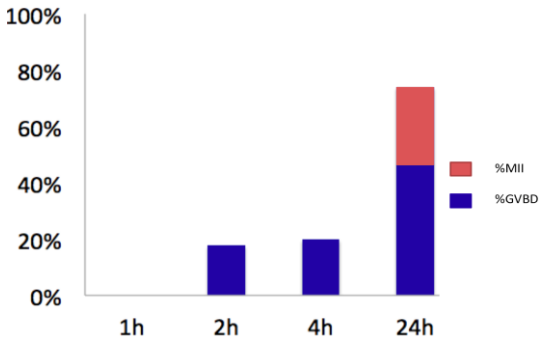

Figure 8. Germinal vesicle breakdown (GVBD) rates after pre-IVM incubation with 25 nM C-type natriuretic peptide (CNP) for 1 hour, 2 hours, 4 hours and 24 hours. COCs were retrieved from unstimulated mice aged 5 weeks old in presence of 25 nM CNP. Data are presented as percentage.

The majority of COCs, following their pre-maturation incubation, were transferred into IVM media and incubated for a further 18 hours (the control group (IVM), was only incubated in IVM media). From the 76 COCs cultured with IVM only, 75% of COCs developed into mature eggs (Figure 9). At the end of this period maturation

rates (as assessed by polar body extrusion) were: IVM (control, n=57; 75%), 1 hour+IVM (n=47, 87%), 2 hours + IVM (n=34, 74%), 4 hours + IVM (n=30,73%) and 24 hours + IVM (n=17, 53%), maturation rates did not vary between pre-IVM groups compared to IVM control group except that there was a notable number of degenerated oocyte leaving around 50% of healthy MIIs oocytes in IVM period (Figure 9, Table 1).

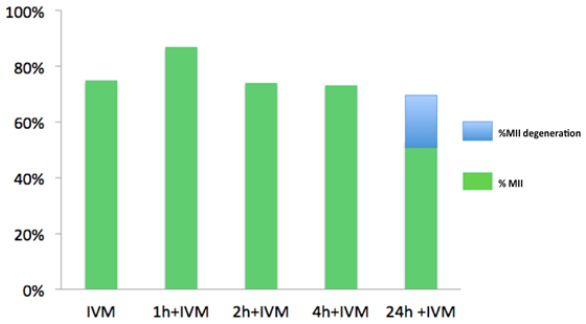

Figure 9. Maturation rate, as evidenced by first polar body (PBI) extrusion, following 1 hour, 2 hours, 4 hours and 24 hours pre-IVM culture in presence of 25 nM CNP plus 18 hours IVM. Data are presented as percentages.

Table 1. Effects of pre-incubation treatment supplemented with C-type natriuretic peptide (CNP) on the embryonic development at day 2 and day 5 following IVF

	#COCs	#MII (%)	#2-cell (%)	#Blasto (%blasto/MII) (%blasto/2-cell)
IVM	76	57 (75%)	32 (56%)[a]	21 (37%) (66%)
1h+IVM	54	47 (87%)	21 (45%)	14 (30%) (67%)
2h+IVM	46	34 (74%)	12 (35%)	5 (15%) (42%)
4h+IVM	41	30 (73%)	13 (43%)	10 (33%) (77%)
24h+IVM	32	17 (53%)	4 (24%)[b]	2 (12%) (50%)
IVF		104	82 (79%)	59 (57%) (72%)

Group	#COCs	#MII (%)	#2-cell/COCs (%)	#2-cell/MII (%)	#Blasto/COCs(%)	#Blasto/2-cell (%)
IVM	76	57 (75.0%)[a]	32 (42.1%)[a]	(56.1%)[a]	21 (27.6%)[a]	(65.6%)[a]
1h+IVM	54	47 (87.0%)[a]	21(38.9%)[a]	(44.7%)[a]	14 (25.9%)[a]	(66.7%)[a]
2h+IVM	46	34 (73.9%)[a]	12 (26.1%)[a]	(35.3%)[a]	5 (10.9%)[a]	(41.7%)[a]
4h+IVM	41	30 (73.2%)[a]	13 (31.7%)[a]	(43.4%)[a]	10 (24.4%)[a]	(76.9%)[a]
24h+IVM	32	17 (53.1%)[b]	4 (12.5%)[b]	(23.5%)[b]	2 (6.3%)[b]	(50.0%)[a]
IVF		104	82 (78.8%)[c]		59 (72%)[a]	

Different letters in one column indicate significant differences (P <0.05). b is different from a (P < 0.05).

Oocyte maturation, fertilization (as assessed by the 2 cell rate) rates and further embryonic development are summarized in Table 1. After 24 hours pre-incubation treatment with 25 nM CNP, oocytes had very poor competences as evidenced by the significantly lower 2-cell rate. From the results, the 2-cell rates were significantly lower in the 24 hours + IVM group, compared to that of IVM control group ($P < 0.05$) (Figure 10). There were no significant differences between the IVM and 1hour + IVM groups in 2-cell rate ($P > 0.05$) (Figure 10). In addition, no differences were seen in the blastocyst formation following 1 hour, 2 hours, 4 hours and 24 hours incubation (Figure 10).

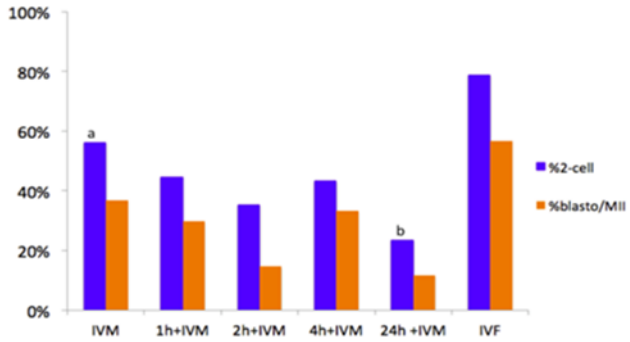

Figure 10. Evaluation of embryo quality after IVF, following 1 hour, 2 hours, 4 hours and 24 hours in the presence of 25 nM CNP condition plus 18 hours IVM. The IVM group was only matured in IVM media for 18 hours. Data are presented as percentages. a and b indicates significant difference where $P < 0.05$. b is different from a ($P < 0.05$)

3.5 Discussion and conclusion

In the preliminary experiment, COCs were derived from unstimulated mice at age of 5 to 6 weeks to investigate the effect of cAMP modulator, CNP, in pre-IVM culture media, which resume oocyte maturation in IVM media. This study was seeking to mimic the processes used in Romero *et al* (2016)'s study as close as possible, in order to test our mouse strain, lab conditions and culture media. In their study they cultured COCs for 48h in pre-IVM media with 25 nM CNP prior to IVM, and this treatment was initially tested in the current experiment, but this treatment was abandoned due to the extensive signs of aging and atresia which was inconsistent with Romero et al. 2016 with low meiotic arrest rates in their study. It was considered the time point of 48 hours Pre-IVM was too long and new shorter time points were established.

GVBD was suppressed completely only in the 1 hour Pre-IVM treatment, the longer pre-IVM periods induced higher chance of GVBD, which negatively impacts on the oocyte reaching metaphase 2 and remaining healthy. Romero et al (2016) has cultured in pre-IVM media for 48 hours, whereas others utilized time points of 12 hours or 24 hours (Downs *et al.*1986), 1 or 2 hours (Albuz *et al.* 2010), 1 hour (Zeng *et al.* 2014) and 24 hours (Wei *et al.* 2017). In addition, the timing of meiotic resumption differed among species that in both two-step and one step IVM, the progression of GV to GVBD induced by hormonal stimulation takes approximately 1 hour to 3 hours in mice (Conti & Franciosi 2018), suggesting any time point over 3 hours may be too long.

The results have shown that the CCs became loosely attached or absent, and 40% of COCs became GVBD while 30% of COCs became MII after 24 hours pre-IVM incubation. This led to a higher percentage of degenerated oocytes entering into 18 hours IVM culture period, post 24 hours pre-IVM. It could be speculated that CNP would act on cumulus cells first then the oocyte via the gap junctions that exist between CCs and the immature oocyte within the follicle. When the cumulus cells surrounding the oocyte become loose, the CNP may not be able to prevent spontaneous nuclear maturation and thus would not assist the nuclear-cytoplasmic synchrony as required.

Although the maturation rates were higher in 24 hours pre-IVM group, the 2-cell and blastocyst rates were significantly lower than IVM group, which indicated that pre-incubation with CNP did not improve the outcomes. These data were contrary to the findings of Romero et al., 2016 which we postulated may be due to subtle differences in mouse strain, age or media components. FSH was included in Romero's study in both pre-IVM and IVM media and this could have led to better oocyte growth compared to the current study which utilized PMSG.

In rodents, PMSG has been found to have both FSH and LH activity on the follicular growth, and with longer half-life compared with FSH (Stewart *et al.* 1976). In addition, either LH or FSH can induce the flow of cAMP in the cumulus cells, the flows diffuse into oocyte via gap junction to maintain oocyte meiotic arrest (Schultz *et al.* 1983; Racowsky 1985). Secondly, the inconsistency of the results may relate to

the different age groups of the animal that were used in the studies. Pre-pubertal mice (19-21 days old) were used in Romero *et al.* (2017) and immature female mice (21-24 days old) were used in Zeng *et al.* (2014), while juvenile mice (35-42 days old) were chosen in the current study. Epping and Schroeder (1989) indicated that the age of mice has direct impact on the quality of oocytes. These studies with better results may be related to the younger mice they have used. Finally, there was single dose of CNP (25 nM) used in the experiment, the different concentrations of CNP were considered useful to be included in the further study to investigate the relationship between CNP dose and meiotic arrest maintenance.

CHAPTER 4

CNP dose rates and time points of pre-IVM efficacy in unstimulated mice between 5-6 weeks old

4.1 Abstract

In vitro maturation (IVM) is a promising assisted reproductive technology (ART) for human infertility treatment. However, when cumulus-oocyte complexes (COCs) are removed from their environment, when manipulated *in vitro*, it leads to a decrease of intra-oocyte cAMP causing spontaneous nuclear maturation and an asynchrony with the oocytes' cytoplasmic maturation, resulting in poor embryo developmental outcomes. Nuclear and cytoplasmic synchrony is important during oocyte maturation within antral follicles. It is maintained partially by the actions of c-type natriuretic peptide (CNP) binding with natriuretic-peptide-receptor 2 (NPR2), supporting high cyclic-adenosine-monophosphate (cAMP) levels thus holding the oocyte in meiotic arrest. It has been reported that the addition of CNP to pre-IVM media has the capacity of maintaining cAMP levels and thus improve synchrony. To investigate whether the cAMP modulator, CNP, can inhibit the initiation of nuclear maturation during pre-IVM period and thus improve final embryo development, COCs were derived from unstimulated ovaries of female mice age around 5-6 weeks old, and either matured directly in IVM media for 18 hours or placed in a pre-IVM media supplemented with 0, 25, 50, 100, 150 and 200 nM CNP for 4 or 24 hours and then matured for 18 hours. Germinal vesicle breakdown (GVBD) was only totally suppressed in the 200 nM CNP pre-IVM treatment for 4 hours (0%). No differences were seen in the maturation rates following 4 hours pre-IVM incubation. Blastocyst rates following 4 hours pre-incubation with 25 nM CNP (73.5%) were significantly higher than the conventional IVM group (45.3%, $P<0.05$). These results suggest that pre-incubation with CNP-supplemented media maintained meiotic arrest in a dose-dependent manner, and the cAMP modulator, CNP did not routinely improve outcomes compared to the conventional IVM protocol in our laboratory.

4.2 Introduction

In vitro maturation (IVM) is a promising assisted reproductive technology (ART) for the treatment of human infertility such as women with polycystic ovary syndrome (PCOS) (Chian *et al.* 2013; Ellenbogen *et al.* 2014). However, IVM has shown poor fertilisation rates in humans compared to those achieved with conventional controlled ovarian hyperstimulaiton (Child *et al.* 2002). One reason proposed for the poor embryo developmental outcomes is due to the asynchronization of nuclear and cytoplasmic maturation (Eppig *et al.* 1994). *In vivo*, increased level of cyclic adenosine monophosphate (cAMP) maintains oocyte meiotic arrest, which allows time for synchronous maturation of the cytoplasm and nucleus prior to the luteinizing hormone (LH) surge (Vaccari *et al.* 2009). In response to the LH surge, lower levels of cAMP enables the nuclear oocyte maturation to resume (Norris *et al.* 2009). However, once immature oocyte are removed from their follicular environment when manipulated *in vitro,* this leads to an instant decrease of intra-oocyte cAMP causing spontaneous nuclear maturation and an asynchrony with the oocytes' cytoplasmic maturation (Pincus & Enzmann 1935; Edwards 1965; Donahue 1968).

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP; also known as natriuretic peptide precursor C) are the most important natriuretic peptides in the mammalian species (Kangawa & Matsuo 1984; Saito *et al.* 1989). The expression of ANP and BNP is mostly dependenton their natriuretic peptide receptor A (NPRA, also known as NPR1). CNP-22 is the version of CNP, which is mainly produced from granulosa cells; it has limited activity and only binds its specific receptor, natriuretic peptide receptor B (NPRB, also known as NPR2), which is primarily expressed by cumulus cells (Potter *et al.* 2006). CNP binding with NPR2 causes an increase in intracellular cyclic guanosine monophosphate (cGMP) levels in the cumulus cells. The high levels of cGMP diffuse into oocyte via gap junction to block phosphodiesterases (PDE3A) activity and maintain intracellular cAMP levels, thereby provide sufficient time for nuclear and cytoplasmic maturation synchrony (Norris *et al.* 2009; zhang *et al*. 2015). It has been reported that the addition of CNP to pre-IVM media has the capacity of maintaining cAMP levels of stimulated/unstimulated F1 mice up to 48 hours, emulating *in vivo* conditions and improving IVM outcomes (Romero *et al.* 2016). However, based on

the preliminary data (Chapter 3), the signs of aging and atresia of oocytes were observed, as well as the significant low maturation rates in 48 hours pre-IVM group supplemented with 25 nM CNP. In addition, there was no difference in the oocyte developmental competence between the different pre-IVM time points.

The purpose of this study was to investigate exposure times: 4 hours and 24 hours to pre-IVM media supplemented with five different concentrations of CNP with respect to maturation rates of germinal vesicles (GVs) derived from compacted cumulus-oocyte complexes (COCs), and also to examine the subsequent fertilization and development of GVs following a pre-maturation period with CNP. It is hypothesized that the different concentrations of CNP supplemented α-MEM medium containing E_2, PMSG and GDF9 will slow down nuclear maturation, thereby reducing the rate of GVBD during either 4 hours or 24 hours pre-maturation period and improve oocyte maturation and blastocyst competency following fertilization compared to oocytes that are not exposed to the pre-incubation period.

4.3 Materials and Methods

The materials and methods of this experiment are as described in Chapter 2.

Experimental design

The experimental design is shown in Figure 11. The total number of 1383 COCs were collected from small antral follicles of 60 unstimulated female mice aged 5-6 weeks and matured either directly in IVM media for 18 hours (IVM control group) or incubated with 0 nM, 25 nM, 50 nM, 100 nM, 150 nM and 200 nM CNP for 4 hours or 24 hours based on the findings that 48 hours were too long in our system prior to IVM (see preliminary data) all at 37°C in humidified 5% CO_2 in air. At the end of the pre-incubation period, cohorts of COCs (approximate one third of total number) were denuded of cumulus cells using a fine glass pipette and assessed for GVBD. The oocytes nuclear status was assessed as described in Figure 6. The cumulus cells surrounding the oocyte were also assessed for signs of expansion as this gives an indication that maturation of the oocyte has been initiated. The remaining compacted COCs were transferred to IVM media for 18 hours. Following IVM, those extruding a

polar body were subjected to IVF. Matured oocytes were incubated at 37°C, 5% CO_2 and saturated humidity with capacitated sperm obtained from C57Bl6/CBA male (concentration 0.25 million motile sperm/ml. After 3 hours, presumptive fertilized oocytes were washed twice and were transferred to 30µl Cook cleavage medium (Cook Medical) under mineral oil in groups of approximately 10 zygotes at 37°C, 5% CO_2 in air and saturated humidity. At 20 hours post insemination, the embryos were assessed for cleavage (2 cells) and this was used as a fertilisation measure. All cleaved embryos were transferred to blastocyst media (Cook Medical) 48 hours later, and observed for blastocyst formation at 72 and 96 hours post insemination. As an *in vivo* control group, COCs were obtained from females aged 5-6 weeks/36 weeks old with an intraperitoneal injection (IP) of 5IU PMSG followed by IP injection of 5IU hCG 48 hours later. The mature COCs were collected from the oviduct 13 hours post hCG and inseminated with the same sperm sample and zygotes were cultured under the same conditions as the IVM oocytes.

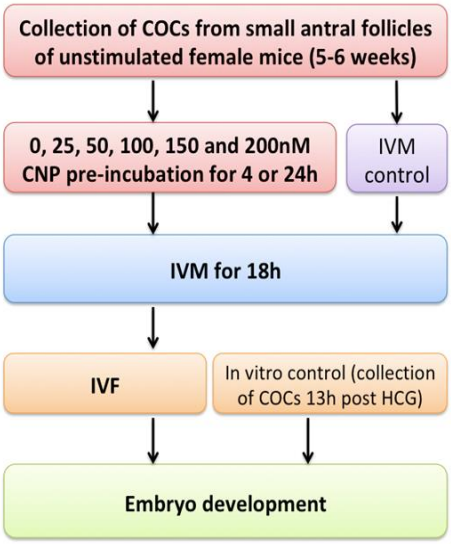

Figure 11. Design for experiment using young mice (5-6 weeks old). 1383 COCs were either matured directly in IVM media for 18 hours (IVM control group) or incubated with all six concentrations of CNP for 4 or 24 hours (treatment groups). At the end of the pre-incubation period the status of the GV was assessed. At the end of the IVM culture period the maturation status of the oocyte was assessed.

Statistical analysis

Fisher's exact or Chi-square test of independence using the GraphPad Prism 5 software program, version 5.0c (GraphPad Software Inc., San Diego, CA, USA) were

used to compare GVBD, maturation status, 2-cell, blastocyst and hatching rates between treatments. The Chi-square test with Yates' correction for continuity was used to compare treatments for numbers of 5 and less, while Fisher's exact test was used for numbers greater than 5. The level of statistical significance was defined as $P < 0.05$.

4.4 Results

All treatments with CNP at 4 hours and 24 hours respectfully, partially inhibited GVBD during the pre-incubation phase compared to 0nM CNP ($P<0.05$, see Figure 12). All five concentrations of CNP used in the study (25, 50, 100, 150 and 200 nM) efficiently inhibited GVBD after 4 hours pre-IVM incubation with 30%, 10%,8%, 2% and 0% of GVBD respectively, compared with 0 nM CNP ($P<0.05$). At the same time point (4 hours pre-IVM), the inhibitory effect of 200 nM CNP was significantly higher than 25, 50, 100 and 150 nM CNP-treated groups ($P<0.05$, Figure 12A). GVBD was only totally suppressed in the 200 nM CNP pre-IVM treatment for 4 hours (0%) (Figure 12A) with longer pre-IVM times and lower concentrations of CNP causing breakdown of the GV at an increasing rate, with up to 90% in the 24 hours pre-IVM period without CNP present (Figure 12B).

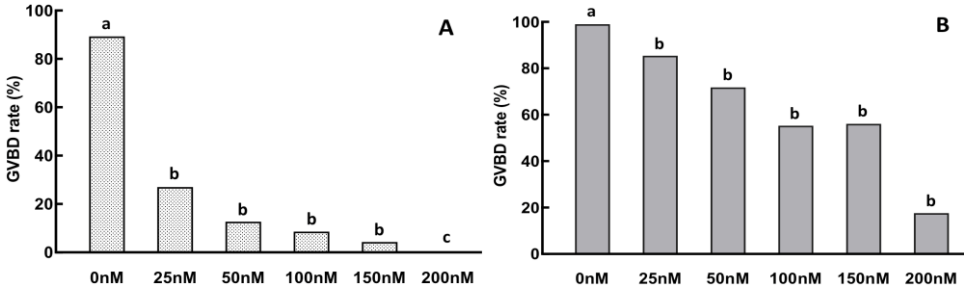

Figure 12. Germinal vesicle breakdown (GVBD) rates after pre-IVM incubation with 0 nM, 25 nM, 50 nM, 100 nM, 150 nM and 200 nM C-type natriuretic peptide (CNP) for 4 hours (A) and 24 hours (B). COCs were retrieved from unstimulated mice aged 5 weeks old in presence of 0 nM, 50 nM, 100 nM, 150 nM and 200 nM CNP. Data are presented as percentage. Different letters indicate significant differences ($P < 0.05$) within the same graph. (A) b is different from a ($P < 0.05$), c is different from a and b ($P < 0.05$). (B) b is different from a ($P < 0.05$).

Maturation rate from the IVM control group was 77.4% (Figure 13). After 4 hours Pre-IVM with 0 nM CNP, oocytes were placed in IVM culture media for 18 hours, only 46.8% of oocytes with polar body extrusion and the rest were fragmented or

remains immature. After pre-IVM incubation with 25, 50, 100, 150 and 200 nM CNP, maturation rates were 68.9%, 67.2%, 73.8%, 78.2% and 74.0%, respectively, significantly higher compared with that in the 0 nM CNP group ($P < 0.05$) (Figure 13A). However, after 24 hours pre-IVM incubation with 100, 150 and 200 nM CNP, maturation rates were 59.2%, 55.7% and 55.8%, respectively, significantly lower than in the IVM control group ($P<0.05$) (Figure 13B). Maturation rates did not differ significantly between treatment groups (25, 50, 100, 150 and 200 nM) following 4 hours pre-IVM incubation compared with the conventional IVM group. However, maturation rates were improved after 4 or 24 hours pre-incubation with different concentrations of CNP compared with 0 nM CNP group. GVBD, polar body extrusion and even atresia had occurred in the majority of oocytes in the 0 nM CNP group, thus they were not further matured.

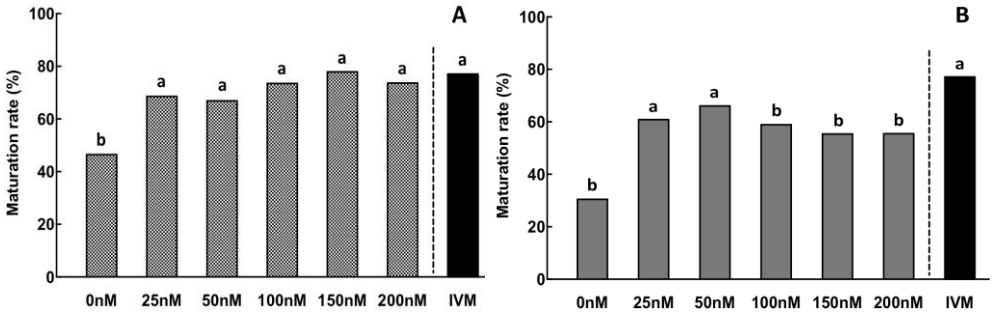

Figure 13. Maturation rate, as evidenced by first polar body (PBI) extrusion, following (A) 4 hours and (B) 24 hours pre-IVM incubation in the presence of 0 nM, 25 nM, 50 nM, 100 nM, 150 nM and 200 nM CNP plus 18 hours of IVM culture. The IVM control group was only matured in IVM media for 18 hours. Data are presented as percentages. Different letters indicate significant differences ($P < 0.05$) for graph A and B ($P < 0.05$).

The 2-cell per MII in the conventional IVM group was 55.2% while at 4 hours pre-IVM with 0, 25, 50, and 100 nM CNP were 40.5%, 53.8%, 45.6%, respectively, there were no significantly differences between IVM and these treatment groups. However, 2-cell rates at 4 hours pre-IVM with 150 and 200 nM (39.5% and 35.2%) were significantly lower compared with the conventional IVM (55.2%, $P<0.05$, Fishers Exact, Figure 14A).

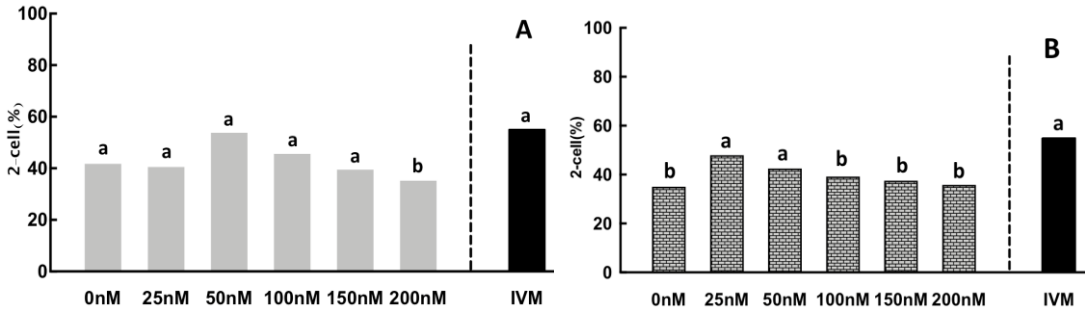

Figure 14. 2-cell rates following 4 hours (A) and 24 hours (B) pre-IVM incubation in presence of 0, 25, 50, 100, 150 and 200 nM CNP plus 18 hours of IVM culture. The IVM group was only matured in IVM media without CNP for 18 hours. COCs were retrieved from unstimulated mice aged 5 weeks old in presence of 0, 25, 50, 100, 150 and 200 nM CNP and fertilized and embryo cultured for up to 5 days. Each column represents the 2-cell stage rate as a percentage of matured oocytes, and the blastocyst formation is expressed as the percentage of 2-cell embryos. Data are presented as percentages. Different letters indicate significant differences ($P < 0.05$) for figures a and b.

Notably, the blastocyst rate per 2-cell following 4 hours pre-incubation in the presence of 25 nM CNP was significantly higher than the conventional IVM group ($P<0.05$, Fishers Exact, Figure 15A), but there was no significant differences compared with the IVF group ($P >0.05$). After 24 hours pre-incubation treatment with 25, 50, 100,150 and 200 nM CNP, oocytes had very poor competences as evidenced by the significantly lower blastocyst rates: 23.5%, 19.4%, 20.7%, 20.8% and 12.5%, respectively compared to the conventional IVM (45.3%, $P<0.05$, Fishers Exact, Figure 15B). 2-cell rates and blastocyst formation following 24 hours pre-IVM incubation in the presence of 100, 150 and 200 nM CNP were significantly lower compared with that of IVM control group ($P<0.05$, Fishers Exact, Figure 14B & Figure 15B).

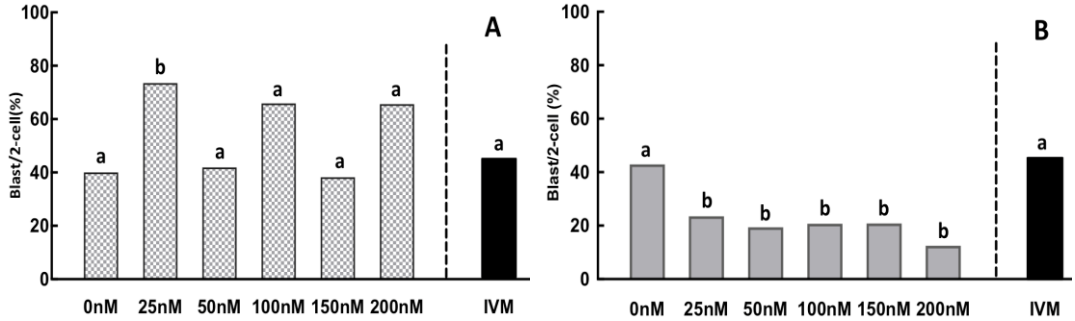

Figure 15. Blastocyst formation following 4 hours (A) and 24 hours (B). Pre-IVM incubation in presence of 0, 25, 50, 100, 150 and 200 nM CNP plus 18h of IVM culture. The IVM group was only matured in IVM media for 18 hours. COCs were retrieved from unstimulated mice aged 5 weeks old in presence of 0, 25, 50, 100, 150 and 200 nM CNP and fertilized and embryo cultured for up to 5 days. Each column represents the 2-cell stage rate as a percentage of matured oocytes, and the blastocyst formation is expressed as the percentage of 2-cell embryos. Data are presented as percentages. Different letters indicate significant differences ($P < 0.05$) for figures a and b.

Table 2. Effects of 4 hours pre-incubation treatment supplemented with C-type natriuretic peptide (CNP) on the embryonic development at day 2 and day 6 following IVF.

Group	#COCs	#MII (%)	#2-cell/COCs (%)	#2-cell/MII (%)	#Blasto/COCs (%)	#Blasto/MII (%)	#Blasto/2-cell (%)
IVM	124	96 (77.4%)^{a}	53 (42.7%)^{a}	(55.2%)^{a}	24 (19.4%)^{a}	(25.0%)^{a}	(45.3%)^{a}
0nM	77	36 (46.8%)^{b}	15 (19.5%)^{b}	(41.7%)^{a}	6 (7.8%)^{b}	(16.7%)^{a}	(40.0%)^{a}
25nM	122	84 (68.9%)^{a}	34 (27.9%)^{a}	(40.5%)^{a}	25 (20.5%)^{a}	(29.8%)^{a}	(73.5%)^{bd}
50nM	119	80 (67.2%)^{a}	43 (36.1%)^{a}	(53.8%)^{a}	18 (15.1%)^{a}	(22.5%)^{a}	(41.9%)^{a}
100nM	122	90 (73.8%)^{a}	41 (33.6%)^{a}	(45.6%)^{a}	27 (22.1%)^{a}	(30.0%)^{a}	(65.9%)^{ad}
150nM	110	86 (78.2%)^{a}	34 (30.9%)^{a}	(39.5%)^{b}	13 (11.2%)^{a}	(15.1%)^{a}	(38.2%)^{a}
200nM	123	91 (74.0%)^{a}	32 (26.0%)^{a}	(35.2%)^{b}	21 (17.0%)^{a}	(23.1%)^{a}	(65.6%)^{ad}
IVF		160	127 (79.4%)^{c}		90 (56.3%)^{b}		(70.9%)^{d}

Different letters in one column indicate significant differences ($P < 0.05$). b is different from a ($P < 0.05$), c is different with a and b ($P < 0.05$), d is different with a and b ($P < 0.05$).

4.5 Discussion

The current study mimicked the recent study of Romero et al. (2016), however, the oocyte maturation rates were significantly lower (0%) in pre-IVM groups supplemented with 25 nM CNP of 48 hours culturing compared to IVM control group based on the preliminary data and the signs of aging and atresia were observed (Chapter 3). It was considered that 48 hours pre-IVM applied to two-step IVM was too long and shorter time points were established (1hour, 2 hours, 4 hours and 24 hours). In addition, the preliminary data did not show any difference between these time points (1 hour, 2 hours, 4 hours and 24 hours), thus, different concentrations of CNP were decided to be investigated in the study. On the other hand, the progression

of GV to GVBD takes approximately 1 to 3 hours in mice with hormonal stimulation *in vitro* without CNP (Conti & Franciosi 2018), 1 hour and 2 hours pre-IVM time point were excluded and extended pre-IVM periods of 4 hours and 24 hours were included to allow sufficient time for nuclear and cytoplasmic synchrony. The results of the current experiment suggested that pre-incubation with CNP-supplemented media maintained meiotic arrest in a dose-dependent manner. The result was consistent with previous studies (Zhang *et al.* 2010, Romero *et al.* 2016, Wei *et al.* 2017). However, for other pre-maturation groups, either 4 hours or 24 hours, no improvements were seen in overall maturation rates and blastocyst formation compared to IVM controls with no pre-maturation, thus suggesting in this study that CNP was not efficiently improving oocyte developmental competence.

In this experiment, COCs were derived from unstimulated mice, the pre-incubation of 24 hours with a dose of 25 nM CNP, was not efficient in stalling nuclear maturation with more than 70% progressing to GVBD, of which 30% had progressed to the fully matured oocyte. After 4 hours pre-IVM incubation in the presence of 200 nM CNP, the surrounding cumulus cells (CCs) were strongly attached to the oocyte, whereas by 24 hours, the CCs were only loosely attached or absent in presence of 25 nM CNP or where pre-incubation did not include CNP (0 nM CNP). Based on a study by Xi *et al.* (2018), CNP had a dose and time-dependent effect on the maintenance of bovine oocyte meiotic arrest with a maximal effect at 200 nM CNP up to 12 hours. The results of the current study suggested that the effect on GVBD in a longer pre-maturation period induced by CNP is dose-dependent with 200 nM CNP being the most efficient inhibitor of GVBD. On the other hand, there was no difference in oocyte maturation rates in 4 hours Pre- IVM with 200 nM CNP besides the 2-cell rates of this treatment group were significant lower compared to IVM control group.

The oocyte maturation rates (55.8%) and 2-cell rates (35.8%) in 24 hours pre-IVM with 200 nM CNP group were significant lower compared to IVM control group, 77.4% and 55.2%, respectively. These results were contrary to Wei et al. (2017)' study, which showed significant higher in oocyte maturation rates (91.32%) and 2-cell rates (50.84%) in 24 hours pre-IVM supplemented with only 50 nM CNP treatment compared to IVM control group with 70% and 29.8%, respectively. Pre-matured oocytes in the extended pre-IVM period may trigger several cellular changes that are

associated with oocyte aging. It has been widely believed that aging causes increases in the production of reactive oxygen species (ROS), which might contribute to oxidative stress (Takahashi *et al.* 2009) and hardening of the zona pellucida (Xu *et al.* 1997). Aging mouse oocytes also cause a loss of normal mitochondrial function (Zhang *et al.* 2011; Lord *et al.* 2013). Thus either of these events may have contributed to the poor fertilization efficiency. Moreover, 2-cell rates and blastocyst formation from 24 hours pre-IVM incubation did not differ between groups except that 4 hours pre-IVM in the presence of 25, 100 and 200 nM CNP gave higher rates than conventional IVM alone. For all other pre-maturation groups (either 4 hours or 24 hours), no improvements were seen in overall maturation rates and blastocyst formation thus suggesting in this study with COCs from unstimulated mice that the cAMP modulator, CNP, was not efficiently maintaining oocytes meiotic arrest.

The current results are inconsistent with Romero *et al*. (2016). The inconsistence may relate to the different age groups of the animal were used in the study. Pre-pubertal mice (19-21 days) were used in Romero et al. while juvenile mice (35-42 days) were chosen in current study. Epping and Schroeder (1989) indicated that the age of mice has direct impact on the quality of oocytes. Older mice have larger diameter of oocytes and acquire more developmental competence which may indicate why there was less GVBD in Romeros's study, even after 48 hours pre-maturation. Additionally, in the current study, the pre-IVM and IVM culture media was supplemented with PMSG hormone instead of FSH, respectively. In rodents, PMSG has been found to have both FSH and LH activity on the follicular growth, and also PMSG has a longer half-life compared with that of FSH (Stewart *et al.* 1976),). In addition, either LH or FSH can induce the flow of cAMP in the cumulus cells, and the flows diffuse into oocyte via gap junction to maintain oocyte meiotic arrest (Schultz *et al.* 1983; Racowsky 1985).

Based on these facts, there were no differences in 2-cell and blastocyst formation in the treatment groups. CNP failed to improve oocyte development competence. In the current study, the GV stage COCs were retrieved from unstimulated ovaries, and IBMX, one of cAMP modulators, was supplemented in the handling media to prevent spontaneous maturation during collection. The results of the study showed that the

blastocyst rates (73.5%) following 4 hours pre-incubation with 25nM CNP were significantly higher than the IVM control group (45.3%).

The significantly increased oocyte maturity and embryo development was found in Zeng et al. (2014)' study which the pre-puberty mice (21-24 days old) with mild equine chorionic gonadotrophin (eCG) stimulation was used. This result was consistent with Wei et al. (2017)' that the mice oocytes were collected 24 hours after PMSG hormone stimulation. In order to minimize the side effects of the hormone stimulation in IVM, Romero et al. (2016) has conducted the study with no hormone stimulation *in vivo* and achieved the increased oocyte maturation rates and embryo developmental competency in young mice (18-21 days old) only. Taking together with these studies, the hormone stimulation and age were found could be the crucial factors that can affect the success of 2-step IVM system. Based on the results from these studies, young women may benefit from 2-step IVM with mild hormone stimulation to achieve pregnancy.

Remarkably, women with advanced maternal age often have poor response to hormone stimulation and the chance of achieving pregnancy is significantly reduced (Lashen *et al.* 1999 &Liu *et at*. 2003). As the 2-step IVM without hormone stimulation has achieved positive results in young model group, this treatment might also suitable for the aged group. However, there was no previous study has been done by using the aging model.

CHAPTER 5

The efficiency of pre-IVM in old mice age 36 weeks

5.1 Abstract

According to the Australian Institute of Health and Welfare, 25% of women who underwent IVF treatment were over 40 years old. The live birth is only 1% in women over age 44. Therefore women with advanced maternal age are good target group to consider the possible benefit of IVM. The synchronous nuclear and cytoplasmic maturation of oocytes *in vitro* is deemed to be a crucial factor determining the oocyte's ability to fertilize and develop normally and thus it is important, when testing a 2-step system, that within the pre-IVM period, the GVBD process is stalled.

The purpose of this *in vitro* study was to investigate various pre-IVM two-step protocols against the standard IVM protocol in aged mice. The current study using an aged mouse model has identified that CNP maintained meiotic arrest in a time and dose-dependent manner. It was found that the higher the concentration of CNP following 4 hours pre-incubation the more efficiently it could inhibit and when as high as 200 nM CNP there was a completely GVBD inhibition. The rate of Day 6 hatching per Day 5 blastocysts in the 50 nM CNP group was significantly higher compared to that of IVM control group. Apart from this finding, pre-incubation with CNP did not improve 2-cell and blastocyst rates.

5.2 Introduction

Advanced maternal age in humans and rodents is a well-known factor that has negative effects on oocyte developmental competence and fertilization rates, and this is presumed to be attributable to both a decreased number of oocytes and poor oocyte quality (Tatone *et al.* 2008, Lord *et al.* 2013). The aged oocytes significantly cause complex cellular and molecular changes in oocytes (Takahashi *et al.* 2009); these biochemical changes may cause chromosomal aneuploidies, which are correlated to a higher abortion rate and decreased implantation rate (Munne *et al.* 1995; Dailey *et al.* 1996; Marquez *et al.* 2000). However, a few studies have shown that aneuploidy is most likely to occur in early stage IVF embryos following hormone stimulation (Santos *et al*. 2010; Rubio *et al*.2010). Therefore, women of advanced maternal age seeking ART are a good target group to consider the IVM. Recent studies in mouse have showed that the aging of oocytes can be the cause of a loss of mitochondrial membrane integrity which has been indicated to a result in the production of cytoplasmic reactive oxygen species (ROS) and increased oxidative stresses (Lord *et al.* 2013, Zhang *et al.* 2011). Excessive levels of ROS are associated with disturbing meiotic arrest in human oocytes and cell death in mouse embryos (Tripathi *et al*. 2009).

To date, the most successful IVM systems have been in the livestock industry using a 1-step IVM procedure. And this has been mimicked in the ART industry but to date with less success. For cattle and sheep, COCs are isolated from the antral follicles of abattoir sourced ovaries and cultured for 24 to 48 hours in IVM medium, which contains common basal IVM medium such as minimum essential medium (MEM) (Sigma Aldrich, Australia) or TCM199 (Medium 199, Sigma Aldrich, Australia), supplemented with macromolecule such as albumin or serum, and hormones such as FSH (Sutton *et al.* 2003). It has been reported that prolonged *in vitro* maturation could deteriorate the quality of oocytes and induce aging of the oocytes during MII arrest period (Kikuchi *et al.* 2000; Agung *et al.* 2006), subsequently compromising embryo developmental competence. While this ‘aging’ is just ‘time in the incubator’, it is unclear whether it has similar pathways of deterioration for oocytes from aged mothers, but perhaps oocytes from aged mothers do have a longer exposure to ROS. In either case, with aged oocytes having lower developmental potential, it is critical

that they get a robust IVM system to give the best possible chance of gaining competent blastocysts for freezing and transfer and the 2-step IVM system is becoming an option (Goud et al. 2008).

The two-step IVM system is a new approach, based on the knowledge of the cyclic nucleotides that involves oocyte meiotic arrest and resumption, and the physiology of how the oocyte interacts with cumulus cells to regulate the levels of cAMP (Romero *et al*. 2016). The two-step IVM system composes of a pre-IVM and an IVM step. Pre-IVM media has relatively high levels of a PDE3A inhibitor, such as CNP or IBMX, designed to inhibit the spontaneous oocyte meiotic resumption upon aspiration of COCs from the antral follicle. The COCs are cultured in pre-IVM media for a prolonged period (4-48 hours), allowing for the maintaining the adequate cAMP concentrations which in turn have been upregulated by FSH. In the IVM culture phase, the PDE3A inhibitor is washed out in order to decrease the levels of cAMP and enable oocyte maturation (Zhang *et al.* 2010; Romero *et al.* 2016)

The cellular changes in the aging oocyte, with regards to the 2-step system, may differ to the younger oocytes, but little is known about this, thus it is still unclear whether the two-step IVM system with the cAMP modulator may rescue aging oocytes during *in vitro* maturation phase. The aim of the current experiment was seeking to investigate whether the dose of cAMP modulator (CNP) required in a pre-IVM culture medium in aged mice is similar to young mice. Base on the experiment on young mice group, GVBD inhibition was significantly efficient in 4 hours pre-IVM with 200 nM CNP. It is hypothesized that with 4 hours pre-IVM with addition of 200 nM CNP will have the same effect on GVBD suppression, thereby, allows enough time for the cytoplasmic and nuclear maturation synchrony to occur compare to the control group where no pre-incubation was performed.

5.3 Materials and Methods

The materials and methods used in this experiment are as described in Chapter 2.

Experimental design

The experimental design is shown in Figure 16. Total number of 1321 COCs were collected from small antral follicles of 120 unstimulated female mice aged 36 weeks and matured either directly in IVM media for 18 hours (IVM control group) or incubated with 0, 25, 50, 100, 150 and 200 nM CNP for 4 hours or 24 hours prior to IVM (Figure 16) all at 37°C in humidified 5% CO_2 in air. At the end of the incubation period, 552 COCs (4 hours pre-IVM) and 659 COCs (24 hours pre-IVM) were denuded of cumulus cells using a fine glass pipette and assessed for GVBD. The oocytes were assessed as described in Figure 5. The remaining compacted COCs were transferred to IVM media for 18 hours. Following IVM, those extruding a polar body were subjected to IVF. Matured oocytes were incubated at 37°C, 5% CO_2 and saturated humidity with capacitated sperm obtained from C57Bl6/CBA male (concentration 0.25 million motile sperm/ml. After 3 hours, presumptive fertilized oocytes were washed twice and were transferred to 30µl Cook cleavage medium (Cook Medical, Bloomington, USA) under mineral oil in groups of approximately 10 zygotes at 37°C, 5% CO_2 in air and saturated humidity. At 20 hours post insemination, the embryos were assessed for cleavage (2 cells) and this was used as a fertilization measure 48 hours later, all cleaved embryos were transferred to blastocyst media (Cook Medical), and observed for blastocyst formation at 72 and 96 hours. As an *in vivo* control group, COCs were obtained from females aged 36 weeks old with an intraperitoneal injection (IP) of 5IU PMSG followed by IP injection of 5IU hCG 48 hours later. The mature COCs were collected from the oviduct 13 hours post HCG and inseminated with the same sperm sample and zygotes were cultured under the same conditions as the IVM oocytes.

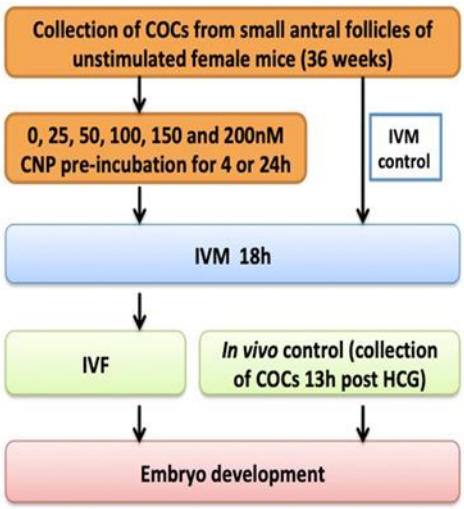

Figure 16. Design for experiment using old mice (36 weeks old). 1321 COCs were either matured directly in IVM media for 18 hours (IVM control group) or incubated with all six concentrations of CNP for 4 or 24 hours (treatment groups). At the end of the pre-incubation period the status of the GV was assessed. At the end of the IVM culture period the maturation status of the oocyte was assessed.

Statistical analysis

The statistical analysis methods are as described in Chapter 4.

5.4 Results

All five concentrations of CNP used in aged COCs (25, 50, 100, 150 and 200 nM) efficiently maintained GVBD after 4 hours pre-incubation (Figure 17A). At the same pre-incubation time, the inhibitory effect of 200 nM CNP was significantly higher compared with 25 and 50 nM CNP that were used in the study ($P<0.05$). COCs pre-incubated with 0nM CNP also inhibited breakdown of the GV, 29.1% of total COCs were underwent the resumption of meiosis (Figure 17A) but this was a significantly higher rate of breakdown than all the CNP treatments. However, COCs with 24 hours pre-IVM time caused breakdown of the GV in increasing numbers. The COCs underwent the meiotic resumption with 25nM dose of CNP was 83.6%, of which 36.1% had progressed to MII. For the group with 0nM CNP, GVBD rate was 99.6%, of which 82.0% had progressed to MII (Figure17B). These results show that 24 hours pre-incubation period caused high portions of GVs were progressing to MII, suggesting pre-IVM period was too long for aging oocytes.

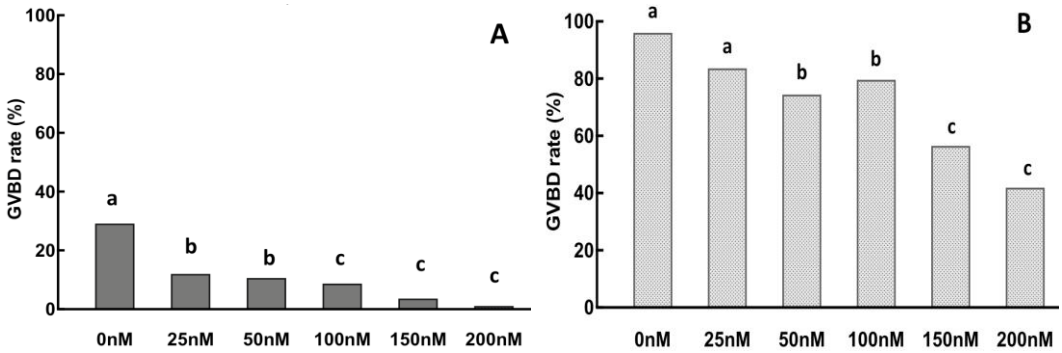

Figure 17. Germinal vesicle breakdown (GVBD) rates after pre-IVM incubation in presence of 0, 25, 50, 100, 150 and 200 nM C-type natriuretic peptide (CNP) for 4 hours (A) or 24 hours (B). COCs were retrieved from unstimulated mice aged 36 weeks old and placed into pre-IVM media supplemented with 0, 25, 50, 100, 150 and 200 nM CNP for 4 or 24 hours. Data are presented as percentage. Different letters indicate significant differences ($P < 0.05$). b is different from a ($P < 0.05$), c is different from a and b ($P < 0.05$).

Maturation rate following CNP supplementation did not show any significant differences between treatment groups (100, 150 and 200 nM) and IVM control group (Figure 18A). Maturation rate was only 28.3 % in the 0 nM CNP group. After 4 hours pre-incubated with 100, 150 and 200 nM CNP, maturation rates were enhanced to 65.6%, 67.8% and 57.7%, respectively, significantly higher compared to the 0nM CNP group ($P<0.05$, Figure 18A). Similarly, after 24 hours pre-incubated with 150 and 200 nM CNP, maturation rates were 49.4% and 36.5%, respectively, significantly higher than 0 nM CNP group (20.0%, $P<0.05$), but significantly lower compared to the IVM control group (64.5%, $P<0.05$, Figure 18B)

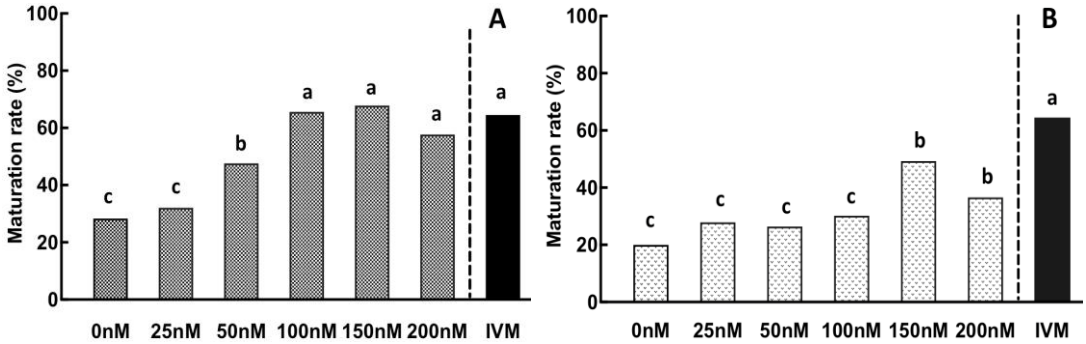

Figure 18. Maturation rate, as evidenced by first polar body (PBI) extrusion, following 4 hours (A) or 24 hours (B) pre-IVM incubation in the presence of 0, 25, 50, 100, 150 and 200 nM CNP plus 18 hours of IVM culture. The IVM control group only underwent 18 hours. Data are presented as percentage. Different letters indicate significant differences ($P < 0.05$). b is different from a ($P < 0.05$). c is different from a and b ($P < 0.05$).

There was no improvement in 2-cell rates in the 4 hours pre-IVM treatment groups supplemented with different concentrations of CNP compared to IVM control group (Figure 19A). Two-cell rate per matured oocytes following 24 hours pre-incubation in the presence of 150 nM CNP (57.6%) was significantly higher than in the IVM control (39.4%) and 0nM CNP group (0%, P<0.05, Figure 19B).

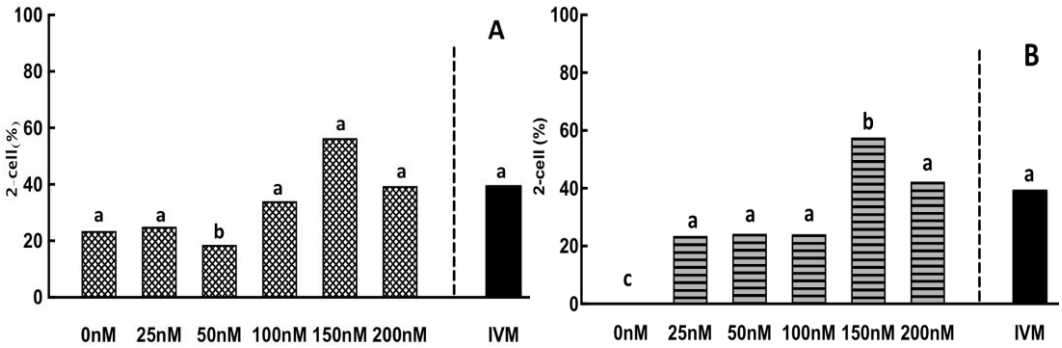

Figure 19. 2-cell rate following a pre-IVM incubation of 4 hours (A) or 24 hours (B) in presence of 0, 25, 50, 100, 150, and 200 nM CNP plus 18 hours of IVM culture. COCs were retrieved from unstimulated mice aged 36 weeks old in presence of 0, 25, 50, 100, 150 and 200 nM CNP and fertilized with resultant embryos cultured for up to 5 days. Results are expressed as 2-cell rate from those matured on day 2. IVM control (without prior pre-IVM incubation) was directly cultured for 18 hours. Data are presented as percentage. Different letters indicate significant differences (P < 0.05) within the same graph. b is different from a (P < 0.05). c is different from a and b (P<0.05).

The blastocyst formation did not differ significantly between treatment groups following 4 hours pre-IVM incubation compared to the conventional IVM control group (Figure 20A). However the rate of Day 6 hatching per Day 5 blastocysts in the 50 nM CNP group was significantly higher compared with that of IVM control group: 14.3% (P<0.05, Table 3). Most of oocytes in the 0 nM CNP group following 24 hours pre-incubation had signs of aging, fragmentation and atresia, thus they were degenerated after the IVM period and not further matured. These results indicate the 24 hours pre-incubation period for aging oocytes was too long, resulting in degeneration of embryos.

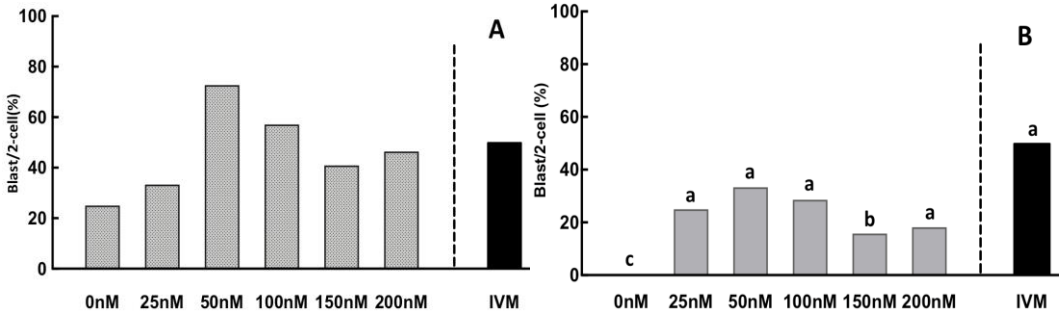

Figure 20. Blastocyst formation following a pre-IVM incubation of 4 hours (A) or 24 hours (B) in presence of 0, 25, 50, 100, 150, and 200 nM CNP plus 18 hours of IVM culture. COCs were retrieved from unstimulated mice aged 36 weeks old in presence of 0, 25, 50, 100, 150 and 200 nM CNP and fertilized with resultant embryos cultured for up to 5 days. Results are expressed as blastocyst rate on Day 5 from those forming 2-cell. IVM control (without prior pre-IVM incubation) was directly cultured for 18 hours. Data are presented as percentage. Different letters indicate significant differences ($P < 0.05$) within the same graph. b is different from a ($P < 0.05$). c is different from a and b ($P<0.05$).

Table 3. Effects of 4 hours pre-IVM treatment supplemented with C-type natriuretic peptide (CNP) on the embryonic development at day 2 and 6 after IVF.

Group	#COCs	#MII (%)	#2-cell/COCs (%)#2-cell/MII (%)	#Blasto/COCs(%)Blasto/2-cell (%)
IVM	76	57 (75.0%)[a]	32 (42.1%)[a] (56.1%)[a]	21 (27.6%)[a] (65.6%)[a]
1h+IVM	54	47 (87.0%)[a]	21(38.9%)[a] (44.7%)[a]	14 (25.9%)[a] (66.7%)[a]
2h+IVM	46	34 (73.9%)[a]	12 (26.1%)[a] (35.3%)[a]	5 (10.9%)[a] (41.7%)[a]
4h+IVM	41	30 (73.2%)[a]	13 (31.7%)[a] (43.4%)[a]	10 (24.4%)[a] (76.9%)[a]
24h+IVM	32	17 (53.1%)[b]	4 (12.5%)[b] (23.5%)[b]	2 (6.3%)[b] (50.0%)[a]
IVF		104	82 (78.8%)[c]	59 (72%)[a]

Group	#COCs	#MII (%)	#2-cell/COCs (%)	#2-cell/MII (%)	#Blasto/COCs (%)	#Blasto/MII (%)	#Blasto/2-cell (%)	#Hatching/Blasto (%)
IVM	110	71 (64.5%)[a]	28 (25.5%)[a]	(39.4%)[a]	14 (12.7%)[a]	(19.7%)[a]	(50.0%)[a]	2 (14.3%)[a]
0nM	60	36 (28.3%)[c]	4 (6.7%)[c]	(23.5%)[a]	1 (1.7%)[c]	(5.9%)[b]	(25.0%)[a]	0 (0%)[c]
25nM	112	36 (32.1%)[c]	9 (32.1%)[a]	(25.0%)[a]	3 (2.7%)[c]	(8.3%)[b]	(33.3%)[a]	0 (0%)[c]
50nM	124	59 (47.6%)[b]	11 (8.9%)[c]	(18.6%)[b]	8 (6.5%)[c]	(13.6%)[a]	(72.7%)[a]	7 (87.5%)[b]
100nM	125	82 (65.6%)[a]	28 (22.4%)[a]	(34.1%)[a]	16 (12.8%)[a]	(19.5%)[a]	(57.1%)[a]	7 (43.8%)[a]
150nM	115	78 (67.8%)[a]	44 (38.3%)[a]	(56.4%)[a]	18 (15.7%)[a]	(23.1%)[a]	(40.9%)[a]	8 (44.4%)[a]
200nM	123	71 (57.7%)[a]	28 (22.8%)[a]	(39.4%)[a]	13 (10.6%)[a]	(18.3%)[a]	(46.4%)[a]	4 (30.8%)[a]

Different letters in a column indicate significant difference where $P < 0.05$. b is different from a ($P < 0.05$). c is different from a and b ($P<0.05$).

5.5 Discussion

The present study aimed to investigate whether the dose requirement of cAMP modulator, CNP, in a pre-IVM culture medium in aged mice is similar to young mice, which improves the quality of aging oocytes matured *in vitro* (Richani et al. 2014). In this study, CNP had a dose and time-dependent effect on the maintenance of meiotic arrest. The result was similar in young mice that CNP arrested meiotic resumption

(chapter 4). However, oocyte maturation quality and blastocyst formation was significantly lower following 24 hours pre-incubation with all concentrations of CNP. Moreover, an extra day of culturing was implemented in the aging mice, partially to delineate more differences in growth rates, allowing the inclusion of blastocyst hatching as a measurement, and slower embryo growth from the aged females was noted.

In the current experiment (old mice aged 36 weeks old), pre-incubation with CNP-supplemented media maintained meiotic arrest in a dose-dependent manner, a high concentration of CNP more efficiently inhibited GVBD, and when as high as 200nM CNP there was a complete GVBD inhibition. CNP treatment did not improve the oocyte maturation and subsequent embryo developmental competences despite effectively inhibit nuclear maturation and no changes were observed in cumulus expansion. The previous studies reported that pre-IVM incubation with cAMP modulator improved the oocyte maturation not only depends on the inhibition of nuclear maturation, but also has a positive effect on COCs metabolism before meiotic resumption (Zeng *et al.* 2013). It would be interesting in future studies to perform metabolite consumption assay following both incubation periods.

In addition, previous studies have confirmed that CNP is produced from granulosa cells, acts via its receptor NPR2 expressed by cumulus cells and supplied to the oocyte through gap junctions to maintain meiotic arrest (Norris *et al.* 2009, Zhang *et al.* 2015). It is assumed that CNP would be acting on cumulus cells first then on the oocyte via the gap junctions which exist between cumulus cells and immature oocyte within the follicle. Cumulus cells are the most crucial components for the oocyte to acquire developmental competence (Gilchrist *et al.* 2008). Vice versa, oocytes also rely on cumulus cells, which are responsible for glucose metabolism and the production of energy by providing pyruvate (Russell & Robker 2007). During oocytes development, aging is one of the factors that affects gene expression of cumulus cells that results in angiogenic genes overexpressing, or it alters energy metabolism pathway, in which aging related insufficient glycolysis production disrupt glucose metabolism in cumulus cells. As a result, the CNP may not be able to prevent spontaneous nuclear maturation and thus not assist in the nuclear-cytoplasmic

synchrony required and in fact, just added to the effect of aging giving negative consequences.

However, even pre-incubation for 4 hours with 0 nM CNP (control group) maintained meiotic arrest in the majority of aging oocytes with only 20% entering GVBD, compared to 80% entering GVBD in young oocytes. This indicated that the aging oocytes might have delayed development compared to young group or that the GVs had lost their ability to breakdown. The longer period of 24 hours incubation was also conducted in the aging oocytes, with result of up to 85% of COCs undergoing nuclear maturation, in which 36% had progressed to Metaphase II that was found in the 25 nM CNP group. A high proportion of these oocytes were degenerate after IVM period with the result of only 20% of healthy matured oocytes. This could be explained by the 24 hours pre-IVM incubation period in the presence of relatively low concentrations of CNP resulting in relatively lower levels of intra-oocyte cAMP, which caused spontaneous nuclear maturation.

Overall, there were few differences in maturation rates, 2-cell and blastocyst rates were observed between treatment groups and control group. It is likely due to the fact that the oocytes collected from aged mice have a slower maturation process and developmental potential due not only to their age but also their health. It was observed in the aged group, that more than 90% of mice had large deposits of fat tissues, and high body weight, which was approximately 1.5 times higher compared to young mice. Some of the ovaries collected from the aged mice appeared hemorrhagic and swollen.

The current study using an aged mouse model has identified that CNP can maintain mouse oocyte meiotic arrest possibly via CCs mediated NPPC/NPP2 pathways. However, the results of the subsequent embryonic development showed that pre-incubation with CNP did not improve 2-cell and blastocyst rates. From these results, it is suggested that 4 hours or 24 hours pre-incubation with CNP does not improve embryonic developmental competence.

CHAPTER 6

Discussion and conclusion

6.1 General discussion and conclusion

The current study mimicked the recent research of Romero *et al.* (2016) to compare the conventional IVM system: one-step IVM and two-step IVM that involves the additional pre-incubation step using cAMP modulator CNP. It is the first report in aging mice that investigates whether two-step IVM system is able to improve the aging oocytes developmental competence. However, significant improvement in oocyte maturation and embryo development was not detected in either young or aging groups.

Based on the results from Chapter 4 (using young mice aged 5-6 weeks old), it demonstrated that only in 4 hours pre-incubation period and with the 4 highest doses of CNP would achieve better results for aged mice. Moreover, an additional day of culturing was implemented in the second experiment, which allowed the inclusion of blastocyst hatching rate as a measurement and delineated more distinct different in embryo growth rates which indicated that embryos from aging mice developed slower compared to young mice. In Chapter 5 (using old mice aged 36 weeks old), pre-incubation with CNP-supplemented media maintained oocyte meiotic arrest in a dose-dependent manner, that is, a high concentration of CNP more efficiently inhibited GVBD, with 200nM CNP totally inhibiting GVBD in all treated COCs. However, even pre-incubation for 4 hours with 0nM CNP (control group) maintained meiotic arrest in the majority of aging oocytes with only 20% of oocytes entering GVBD, compared to 80% of oocytes entering GVBD in experiment in Chapter 4. The results assume that the aging oocytes might have delayed development compared to young group. Different strategies may be required for aging oocytes in the further study, such as addition of incubating time or supplementation of melatonin. Overall, there were few differences in maturation rates, 2-cell and blastocyst rates were observed between the treatment groups and the control group. This might be due to the fact that the oocytes collected from aged mice have a slower maturation process and developmental potential which was not only related to their age but also their health

conditions. It was observed in the aged group, that more than 90% of mice had large deposits of fat tissue, and their body weights were approximately 1.5 times higher compared with the body weights in the young group. Moreover, some ovaries collected from aged mice appeared hemorrhagic and swollen.

The study using an aged mouse model in Chapter 5 has identified that CNP can maintain mouse oocyte meiotic arrest via CC mediated NPPC/NPP2 pathways. However, the results of the subsequent embryonic development showed that pre-incubation with CNP did not improve 2-cell and blastocyst growth outcomes. From these results, it suggested that 4 hours or 24 hours pre-incubation with CNP has not improved embryonic developmental competence.

Although CNP acted in a dose-dependent manner in experiment using young mice and older mice, GVBD was still suppressed in the presence of 0 nM CNP for 4 hours pre-incubation period in the aging group. This age related observation may affect the normal development of the oocyte, which lags, compared with that of young mice group. In addition, mice that were 36 weeks old have similar reproductive function to the aging woman who's approaching menopause. It is well known that advanced maternal age affects hormonal stimulation and disturbs metabolism.

From the experiments in Chapter 4 & 5, it has shown that the cAMP modulator, CNP, regulates the concentration of cAMP by inhibiting GVBD for an extended period during two-step IVM. Based on the experiments in this study, we confirm that CNP works on a dose dependent manner on young and aging COCs. If this study is applied to human clinical IVM, 4 hours pre-incubation period with the presence of 25 nM CNP would be the better option to achieve better clinical outcomes based on results of this experiment. Sánchez *et al*. (2017) also confirmed that 2-step pre-IVM was applied to PCOS patients which achieved significantly higher blastocyst rates compared to IVM control group. The COCs were cultured in pre-incubation for 24 hours supplemented with 25 nM CNP followed by 30 hours IVM culturing and result in significant high maturation (70%) and blastocyst rates (18%) compared to IVM control group, 48% and 8%, respectively. The reasons are as the followings. First, to avoid potential negative effects of CNP, and altering other CNP signaling in the body, for an example, CNP also plays a critical role in maintaining vascular and cardiac

homeostasis by modulating fluid and electrolyte balance and vascular tone (Lumsden *et al*. 2010). Secondly, aging must be considered (two-step IVM system may not be required for women at advanced maternal age). Finally, CNP has the shortest half-life with 2.6 minutes in humans (Potter 2015) and 0.5 to 4 minutes in mice (Ruskoaho 1992). Optimal quality of aging mice can be achieved by introducing more space in cages allowing increased activities along with regular bodyweight monitoring.

In the current study, E2 is required to maintain the competence of cumulus cells to undergo expansion. COCs cultured without E2 supplementation resulted in reduced cumulus expansion and decrease cumulus expansion expression levels (Sugiura et al. 2010).There are two pathways that cross-communicating each other lead to oocyte maturation and COCs developmental competency, endocrine hormones, E2, and paracrine growth factiors, GDF9 (Varnosfaderani et al. 2013 & Sugiura et al. 2010). In addition, There are few studies have proved that CNP could inhibit meiotic resumption and provide sufficient time for the cumulus cells through gap junction to synchronise nuclear and cytoplasmic maturation during pre-IVM phase (Tsuji et al. 2012; zhang et al. 2015; Romero et al. 2016). GDF9 and E2 plays crucial role in cumulus cell expansion and also contribute to further development of oocyte. Thus the results of maturation rates are considered to be related to the cumulative pre-IVM and IVM periods.

The inhibition of nuclear maturation for an extended period during IVM culture is determined as a key factor of the oocytes' maturation and subsequent successful fertilization. However, oocytes developmental competence of IVM showed poor outcomes compared to that of the *in vivo* matured oocytes. Aging oocytes following 24 hours pre-incubation may cause high level of oxidative stress, which is associated with disturbing meiotic arrest in nuclear maturation, and also results in the production of ROS in the cytoplasmic maturation. Therefore, cell death in aging mouse embryos was observed in 0 nM CNP with 24 hours pre-incubation period. Although there were significant outcomes of young mice cultured with 25nM CNP following 4 hours pre-incubation and 150 nM CNP following 24 hours pre-incubation in aging mice, the morphology of spindles and chromosomes need to be investigated in the future study Alternative pre-IVM is not only supplemented with cAMP modulator, antioxidants may also need to be investigated.

Although CNP and IBMX are both powerful cAMP modulators, they active in the different pathways to increase intra-oocyte cAMP concentration, thereby, inhibit spontaneous nuclear maturation (Vigne 1994). The PDE enzymes are composed of 11 different families of subtypes (PDE1-11), which regulate different cellular mechanisms (Nicholson et al. 1991). It has been found that PDE3A are located in the oocyte and PDE4A are located in the granulosa cells, and only PDE3A are responsible for the regulation of cAMP levels in intra-oocyte that inhibit spontaneous nuclear maturation (Thomas et al 2002). Although PDE4A has no direct effects on oocytes spontaneous meiotic resumption, it does mediate cAMP levels in both granulosa and cumulus cells.

Therefore, IBMX, which is a nonspecific PDEs inhibitor (Thomas et al. 2002), is the best cAMP modulator that efficiently to prevent the actions of two types of PDE (PDE3A and PDE4A) which either can alter cAMP formation after manually retrieval of COCs. On the other hand, CNP binds to its receptor NPR2, which is located on both cumulus and granulosa cells, to increase cGMP concentration which leads to PDE3A activity inhibition. CNP, specific PDE inhibitor is used in pre-IVM media.

These results were found not consistent with previous studies, the results are related to the cumulative pre-IVM and IVM periods. During pre-IVM period with the supplementation CNP maintain cAMP levels thus improve nuclear and cytoplasmic synchrony, which is important for oocyte maturation and further embryo developmental competence. During IVM culture, oocytes initiate meiotic resumption and are capable of reaching MII phase with extrusion of the polar body. The result of maturation rate is considered to be related to the cumulative pre-IVM and IVM periods. However, the quality of matured oocyte cannot be determined all with good quality, which poor quality might result in retarded or abnormal 2-cells development may due to spindle, mitochondrial distribution abnormality or other parthenogenesis (Nagai et al. 2006 & Wei et al. 2017).

However, the subtle variations in the protocols, even within the same species would suggest caution before clinical translation. From this study, it has shown that when the conventional IVM works well with successful pregnancy, the minor difference in

cytoplasmic and nuclear synchrony may not have a significant impact overall in unstimulated mice. It should be noted that clinical IVM is often associated with mild *in vivo* stimulation and perhaps the 2-step approach may still be a good option for this situation, rather than changing to a pure *in vitro* maturation system. Although classed as pseudo-IVM these have shown promising results (Ellenbogen et al. 2014).

6.2 Overall conclusions of the thesis

Pre-incubation with CNP-supplemented media in a dose and time-dependent manner has impact on maintaining oocyte meiotic arrest regardless of oocyte age, but did not improve the outcomes compared to the use of conventional IVM protocol in our laboratory. For all other pre-maturation groups (either young or aging), no improvements were seen in overall maturation rates and blastocyst formation, thus it is suggested that in this study the cAMP modulator, CNP, did not efficiently maintain oocytes meiotic arrest in COCs from unstimulated mice. This result demonstrated that the successful nuclear maturation only is unable to enhance the maturation ability of IVM oocytes, asynchronization of nuclear and cytoplasmic maturation may not significantly impact on the lower rate of IVM oocytes.

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Appendix

The composition of media used in Chapter 3 and 4 for COCs collection and culture of COCs isolated from unstimulated ovaries.

Components	Collection Media	Basal IVM Media	IVM Media	Pre-IVM Media
[1] L-15				
[2] FBS, 10% (V/V)				
[2] FBS, 2.5% (V/V)				
[3] Penicillin, 100IU/mL				
[3] Streptomycin, 100μg/mL				
[4] IBMX, 200μM				
[5] α-MEM				
[6] ITS				
[7] r-EGF, 4ng/mL				
[8] EREG, 100ng/mL				
[9] PMSG, 2.5mIU/mL				
[10] CNP				
[11] E_2, 10nM				
[12] GDF9, 50ng/mL				

[1] Leibovitz's L-15 medium, Life Technology, Victoria, Australia
[2] Fetal bovine serum, Life Technology, Victoria, Australia
[3] Penicillin-Streptomycin, Sigma, Victoria, Australia
[4] IBMX, Sigma, Victoria, Australia
[5] α-minimum essential medium, Life Technology, Victoria, Australia
[6] ITS: Insulin-Transferrin-Selenium; at 5ng/mL insulin, 5μg/mL apo-transferrin and 5ng/mL sodium selenite, Sigma, Victoria, Australia
[7] r-EGF, Roche, Minnesota, USA
[8] EREG, R&D Systems Europe, Minnesota, USA
[9] Folligon, MSD-animal-health, Australia
[10] CNP-22, Phoenix Europe, California, USA
[11] 17-β-estradiol, Sigma, Victoria, Australia
[12] GDF9, R&D Systems Europe, Minnesota, USA