EVALUATION OF CONTRACEPTIVE PROPERTIES OF CILOSTAZOL (A PHOSPHODIESTERASE 3A INHIBITOR) IN MICE

A Dissertation

by

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ABSTRACT

The pharmacological development of non-steroidal contraceptives has yet to be achieved. Arresting oocyte maturation without blocking ovulation has been evaluated using different inhibitors of the phosphodiesterase 3A (PDE3A). Unfortunately, PDE3A is also expressed in the heart and blood vessels, and inhibition of PDE3A in oocytes can produce cardiovascular side effects. We reviewed the literature on available PDE3 inhibitors and selected cilostazol (CLZ), which is an FDA approved therapeutic. CLZ has the ability to decrease cellular adenosine uptake and consequently antagonizes side effects of PDE3A inhibition in vital organs. CLZ inhibited oocyte meiotic maturation *in vitro*. CLZ has more degenerative impact on arrested oocytes than matured oocytes, indicating that prolonged meiotic arrest of oocytes is harmful. Administration of CLZ any time from 9h before the ovulatory stimulus to 4h after the stimulus resulted in ovulation of immature oocytes. Controlling CLZ dose, time of CLZ administration, and time of oocyte collection resulted in ovulation of oocytes at different meiotic stages. Oral administrations of CLZ in naturally cycling mice were also observed to block pregnancy whereas remating of those previously treated females resulted in normal offspring and litter sizes. Therefore, CLZ does not only have a wide margin of contraception but also is reversible.

Ovulated immature oocytes were observed to have higher rates of advanced chromatin configuration and cortical granule distribution, normal spindle and chromosomal organization, maturation, and *in vitro* fertilization (IVF) than ovarian immature oocytes. Ovulated metaphase I oocytes that were matured *in vitro* or *in vivo* had higher IVF rates than ovulated mature oocytes. Ovulated germinal vesicle (GV) oocytes that were *in vitro* matured also showed higher IVF rates but when *in vivo* matured, they had lower IVF rates than ovulated mature oocytes because of the high degeneration and low fertilization rates associated with *in vivo* maturation of GV oocytes.

In summary, CLZ merits further evaluation as a non-steroidal contraceptive and is capable of producing oocytes of various meiotic stages with advanced developmental features.

DEDICATION

This study is dedicated to the memory of Badeh M. Alwatar, 1979-2002. Badeh was an intellectual phenomenon, a noble pharmacist, and an amiable and decent friend.

ACKNOWLEDGEMENTS

I cannot find words to express my greatest gratitude to my lord 'Allah' for giving me the strength and the insight to conduct this research, to my teachers and professors who guided me during my learning career, and to my family and friends who supported me.

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Finally, I present my sincere thankfulness to my wife Hope F. Mohammed-Albarzanchi for her devotion, tireless encouragement, cheering me

and standing by me at all times. I am thankful to her for her highly synchronized and fully competent oocytes that produced my beloved offspring.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

In spite billions of dollars spent on contraceptive development, there are still pockets of overpopulation all over the world. According to the international data base of the US census bureau, it is believed that the highest populated country will be shifted from China to India by 2050. While it required 123 years (1804-1927) for the population to produce the 2nd billion, it took 33 years to produce the third billion (from 1927 to 1960) and then only 12-14 years to produce one billion after 1960. The current world population reached seven billion on October 31st, 2011, and is believed to project to 10.1 or 15.8 billion in 2100 when medium or high projection variants are referenced, respectively (United Nations Population Division 2002 and 2011). This intense population growth threatens the limited natural resources of food and fresh water on earth to sustain this massive population increase. This threat is not only haunting increasingly wealthy third-world countries but is also threatening industrialized countries. Numerous factors encourage development of more efficient, non-invasive, safe, cheaper, and convenient birth control. Among those factors are environmental problems such as global warming and desertification, pollution such as the 3 months oil flow disaster in Gulf of Mexico in 2010 and the radioactive materials release accident of the Fukushima Nuclear Power Plant after the Tsunami in

Japan in 2011, population explosion of feral dogs, cats, and hogs that threaten livestock and crops, and political conflicts.

During the 1920s, an Austrian scientist, Ludwig Haberlandt (1885 -1932), reported that administration of ovarian extracts in experimental animals produced temporal sterility. Haberlandt successfully analyzed some previous observations, which linked the development of the corpus luteum to lack of ovulation during pregnancy, into the concept of oral contraceptive pills (Djerassi 2009; Goldzieher and Rudel 1974; Haberlandt 2009; Muller-Jahncke 1988). Currently, there are 2 types of oral contraceptives; the combined pill (combinations of an oestrogen, which is basically ethinyl estradiol and progestogen [synthetic progesterone]) and the progestogen only pill. The pharmacological mechanism of these pills uses the negative feedback loops of estrogen and progesterone on follicle stimulating hormone (FSH) and luteinizing hormone (LH) release, respectively (Berne *et al*. 2004; Odell and Molitch 1974; Kim and Wasik 2011). Studies have found that the current steroidal contraceptive medications precipitate side effects such as venous and arterial thromboembolism, hypertension, ischemic stroke. These medications were also reported to associate with breast cancer (Atthobari *et al*. 2007; Beaber *et al*. 2012; Blanco-Molina *et al*. 2012; Hickson *et al*. 2011; Iatrakis *et al*. 2011; Tanis *et al*. 2001; White *et al*. 1994).

In 1934, Pincus and Enzmann stated that "*maturation of the ovum can be obtained simply by isolating it from the normal follicular environment"* (Pincus

and Enzmann 1934). An inhibitory role for 3'-5'-cyclic adenosine monophosphate (cAMP) on spontaneous maturation was suggested in 1974 by Cho and coworkers after observing *in vitro* inhibition of oocyte meiotic maturation caused by dibutyryl cAMP and theophylline (Cho *et al*. 1974). The Phosphodiesterase 3A (PDE3A) hydrolyzes intraoocyte-cAMP and triggers meiotic maturation. Wiersma and coworkers successfully blocked pregnancy when they administered some PDE3 inhibitors (PDE3-Is) in superovulated and naturally cycling rodents (Wiersma *et al*. 1998). Similar results were obtained with non-human primates (Jeffrey *et al*. 2010; Jensen *et al*. 2005 and 2008). Unfortunately, an adverse effect of tachycardia was observed in both species (Jeffrey *et al*. 2010; Wiersma *et al*. 1998). Inhibition of the PDE3A enzyme in oocytes is believed to inhibit the same enzyme in the heart, vascular smooth muscles, and platelets. No further studies using this novel approach of targeting the final maturation phase of oocytes without interfering with the hormonal profile and ovulation were conducted *in vivo*.

In this study, we considered all available PDE3-Is and selected cilostazol (CLZ) that appears to be a weak (based on IC_{50}) and safe compound in comparison to other PDE3-Is. CLZ was reported to inhibit both cellular PDE3A and cellular uptake of adenosine. Inhibition of cellular adenosine uptake is believed to counterbalance some of the cardiovascular side effects that appear when other PDE3-Is are administered (Liu *et al*. 2000 and 2001). Moreover, CLZ is an FDA (pletal®) approved therapeutic agent that is prescribed to treat people

with intermittent claudication disease, a state that is characterized by lower limb pain while walking. Interestingly, CLZ has not been evaluated for its ability to inhibit oocyte maturation except for the manufacturer studies that found that CLZ does not impair fertility in rats using their methods (Otsuka America Pharmaceutical Inc. 2007).

Two objectives in the current study were evaluated. These are 1) CZL ability to inhibit fertility as a non-steroidal contraceptive agent 2) CLZ ability to improve fertility through improving oocyte quality and fertility). Therefore, experiments were conducted to evaluate CLZ capability of inhibiting oocyte maturation *in vitro* and *in vivo*, define the effect of different times of oral administration of CLZ on oocyte meiotic maturation *in vivo*, test the ability of the *in vivo* arrested oocytes to undergo maturation *in vitro* or *in vitro* and *in vitro* fertilization (IVF), synchronize meiotic and cytoplasmic maturation of oocytes, and synchronize progression of meiotic events among oocytes.

CHAPTER II

IN VITRO EVALUATION OF A PHOSPHODIESTERASE III INHIBITOR, CILOSTAZOL, ON MOUSE OOCYTE MEIOTIC MATURATION AND MORPHOLOGY

Overview

Cilostazol has not been evaluated as an oocyte PDE3A inhibitor. The compound is FDA approved for patients with intermittent claudication. In order to study the inhibitory effects of CLZ on oocyte maturation, antral oocytes were retrieved from hyperstimulated mice and denuded before allocating to 10 different CLZ concentrations (0.00, 0.264, 0.528, 1.057, 2.114, 4.229, 8.458, 16.916, 33.832, and 67.664µM). Subsequently, the oocytes were assessed for maturational and morphological status after 24 and 48h of incubation. CLZ at concentrations of 1.057-67.664µM (24h) and concentrations of 1.057-8.458 µM (48h) arrested oocytes at the germinal vesicle (GV) stage when compared to other concentrations ($P< 0.0001$). A considerable selective degenerative impact of CLZ on GV arrested oocytes was observed in 33.832 - 67.664µM (24h) and 2.114- 67.664µM (48h) when compared to other tested CLZ concentrations ($P<0.05$ and $P<0.0001$, respectively). The degenerative pattern was analyzed and vacuolated oocytes were found to be predominant at low and medium CLZ concentrations, while both vacuolated and fragmented oocyte types were observed at high CLZ concentrations. It is concluded that CLZ can block *in vitro*

oocyte meiotic maturation efficiently and that prolonged maintenance of denuded oocytes at the GV stage is harmful, since mainly GV arrested oocytes were sensitive to the degenerative impact of CLZ. Furthermore, this degeneration increased as the CLZ concentration and incubation time increased, suggesting a role for cAMP in this degeneration. Our study shows that CLZ has a dose dependent inhibitory effect on oocyte spontaneous maturation.

Introduction

Oocyte meiotic maturation occurs during the first meiotic reduction and it represents nuclear development from a fully grown, non-fertilizable, prophase I oocyte to a fertilizable metaphase II (MII) ovum. This meiotic division is accompanied by a characteristic morphological transformation from a meiotically immature oocyte with a germinal vesicle into a mature ovum with the first polar body. Interestingly, oocyte meiotic maturation can take place spontaneously *in vitro* upon oocyte isolation from its follicle or under the surge of LH in preovulatory follicles (Ayalon *et al*. 1972; Donahu 1968; Hutt 2007; Pincus and Enzmann 1935; Wassarman *et al*. 1976). The ability of mammalian oocytes to undergo this spontaneous maturation *in vitro* or a hormonally triggered maturation *in vivo* is mainly due to mechanical interruption or hormonal phosphorylation, respectively, and subsequent closure of numerous membrane gap junctions. This junctional system furnishes the follicular oocytes with meiotic

inhibitory signals such as cAMP and 3'-5'-cyclic guanosine monophosphate (cGMP). Oocytes are capable of synthesizing their own cAMP, but surrounding somatic cells are believed to provide high intraoocyte cAMP levels and to maintain a non-functional state of PDE3A in oocytes; the enzyme that controls meiotic maturation (Gilula 1978; Norris *et al*. 2008 and 2009; Sela-Abramovich *et al*. 2006 and 2008; Sherizly *et al*. 1988). Many investigators have utilized PDE3-Is in experiments of oocyte maturation in order to disclose the biochemical and molecular biological mechanisms that influence meiotic maturation. Also, PDE3-Is have been studied to develop non-steroidal contraceptives that target this final maturational phase “meiotic maturation” of oocytes in humans and animals.

Strong evidence for the predominance of PDE3 on the regulation of oocyte meiotic maturation was furnished by Masciarelli *et al*. (2004). They found that PDE3A knockout mice were capable of ovulating GV oocytes that failed to undergo spontaneous maturation *in vitro* for up to 48h. Moreover, microinjection of PDE3 into GV arrested oocytes cultured in the presence of PDE-I overcame the inhibitory effect of PDE-I on meiotic maturation (Bornslaeger *et al*. 1986). Many PDE3-Is like cilostamide, Org 9935, milrinone, pimobendan, and trequinsin have been studied in numerous species and found to arrest oocyte meiosis at the GV stage by maintaining elevated oocyte cAMP concentrations (Bilodeau-Goeseels 2003; Grupen *et al*. 2006; Jensen *et al*. 2002; Nogueira *et al*. 2005a and b; Thomas *et al*. 2002; Wiersma *et al*. 1998).

Cilostazol is a PDE3A inhibitor that is prescribed for patients with intermittent claudication disease (Chapman and Goa 2003; Meru *et al*. 2006). Although the majority of the PDE3-Is have been used to arrest oocyte maturation, CLZ has not been evaluated. Interestingly, CLZ has an additional unique pharmacological mechanism over the rest of the PD3-Is in that it has the ability to decrease the cellular uptake of adenosine (Liu *et al*. 2000 and 2001; Sun *et al*. 2002). The latter effect is thought to be responsible for antagonizing the side effects that could appear when other PDE3-Is are administered to humans or other mammals, especially for long periods of time (Liu *et al*. 2000). The primary aim of this research was to evaluate the inhibitory effect of CLZ on oocyte meiotic maturation *in vitro*. This compound appears to be the safest of the PDE3-Is; thus it is a suitable candidate for studying inhibition of oocyte meiotic maturation *in vivo* and its potential as a non-steroidal contraceptive in humans and animals.

Materials and methods

Animals

Swiss Webster albino mice, 4-6 weeks of age were purchased from Harlan Laboratories (Houston, TX) and housed in groups of 5-8 mice per cage in the Texas A&M University Laboratory Animal Facility. Mice were under controlled temperature (23°C) and light/dark (14/10 h) cycle. Food and water were provided *ad libitum*. The experiments were conducted in accordance with the

Animal Care Guidelines of the Institutional Animal Care and Use Committee of Texas A&M University.

Reagents

CLZ was purchased from LKT Laboratories, Inc, (St. Paul, MN, USA), Leibovitz's L-15 Medium (1X), liquid, GIBCO® brand, was purchased from Invitrogen (Carlsbad, CA), and EmbryoMax® FHM, liquid, without phenol red was purchased from Millipore (Billerica, MA, USA). Pregnant mare serum gonadotropin (PMSG), hyaluronidase from bovine testes, and dimethyl sulfoxide (DMSO) were all purchased from Sigma Chemical Co. (St. Louis, MO). CLZ was dissolved in DMSO and diluted with overnight CO_2 equilibrated FHM medium to make 10 CLZ concentrations: T1: 0.00 [control group], T2: 0.264, T3: 0.528, T4: 1.057, T5: 2.114, T6: 4.229, T7: 8.458, T8: 16.916, T9: 33.832, and T10: 67.664 µM CLZ. The highest DMSO concentration was at T10 (0.1%) while the minimum DMSO concentration was at T2 (0.003%). The control culture medium contained 0.1% DMSO to detect any possible effect that DMSO could have on oocyte meiotic maturation and/or morphology.

Oocyte collection and incubation

Female mice were injected i.p. with 5 IU of PMSG (0.1ml) to stimulate development of multiple follicles. Forty-seven to fifty hours later, the anesthetized animals were sacrificed by cervical dislocation and the ovaries

were collected in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The antral follicles were punctured under a stereomicroscope (Nikon, SMZ 1500 model) using 30-gauge needles. Cumulus-enclosed oocytes (CEO) were denuded by placing the CEO in Leibovitz's L-15 medium with 1% hyaluronidase for less than 5 minutes with mechanical manipulation. Denuded oocytes (DO) at the GV stages were washed in fresh Leibovitz's L-15 medium to remove the hyaluronidase followed by another two washes in FHM medium before pooling and allocating to each of the CLZ test concentrations. The DO were incubated up to 48h at 37ºC with 5% CO_2 and in a humidified environment. The oocytes were scored at 24h and 48h of culture. No PDE-Is were used in the initial collection and washing steps to exclude any possibility of PDE3-I effects on meiotic maturation and morphology rather than that of CLZ. The isolation procedure from the time antral follicles were punctured to allocation into the treatment groups was approximately 15 minutes per mouse.

At the end of each incubation period, the oocytes were examined for their meiotic status by a stereomicroscope or inverted microscope (Olympus, IX71 model) and classified as follows: 1) Oocytes showing a nuclear membrane and intact nucleolus were classified as meiotically arrested at the GV stage. 2) Oocytes showing no nuclear structures or envelopes were classified as having undergone germinal vesicle breakdown (GVBD). 3) Oocytes that had undergone GVBD but did not have a polar body were classified as metaphase I (MI). 4)

Oocytes that had no nuclear envelopes and with first polar bodies were classified as MII oocytes (Tsafriri and Kraicer 1972). Morphological appearance of oocytes, whether in GV, MI, or MII, was classified as follows: 1) Degenerated/ fragmented: coarse granular, non-homogeneous, and dark (brownish-blackish) cytoplasm; 2) Degenerated/ vacuolated: non-spherical and with or without large perivitelline space oocytes (irregular shape); 3) Non-degenerated oocytes: spherical in shape, with smooth granular and transparent cytoplasm.

Statistical analysis

Multiple linear correlations were used to determine the linear relationship between the treatments and different oocyte stages of meiotic maturation and normality. Correlation was also used to determine the relationship between different oocyte stages starting from T7 (24h) and T4 (48h). Those concentrations were found to represent the concentrations at which the biphasic effects of CLZ on GV type oocytes were shown. The Chi-square test was used to evaluate all other differences. Statistical differences were considered significant at the level of $P<0.05$ using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). At least 3 oocyte replicates were used for each treatment. Each replicate represented oocytes from 3-5 female mice.

Results

Twenty four hour incubation

Oocytes arrested at the germinal vesicle stage

Increasing CLZ concentrations linearly correlated with arresting spontaneous oocyte maturation at the GV stage (r=0.77, P<0.0001, Fig. 2.1). CLZ concentrations of T4-T10 were able to arrest more oocytes at the GV stage than the control and low CLZ concentrations of T2-3 (P<0.0001).The T4-10 concentrations maintained more oocytes at the GV stage than the GVBD stage (P<0.0001). Moreover, T6-8 concentrations were more effective in maintaining oocytes at the GV stage than T4-5 (P<0.0001), indicating a dose response pattern. The GV arresting effect was not consistent at every concentration as higher concentrations of T9-10 showed less GV arrested oocytes when compared to lower concentrations of CLZ of T6-8 (P<0.03) and T10 was not significantly different from T4-5 (P=0.06). To analyze this inability of high concentrations of CLZ to preclude spontaneous maturation at the GV stage, multiple correlation analysis revealed a negative linear relationship between arrested oocytes at GV and degenerated oocytes at concentrations T7-T10 (r=-.0.781, P<0.0001). There was no significant correlation between oocytes that had undergone GVBD and degeneration (r=-0.082, P=0.64), indicating that the GV oocytes are the only oocytes that are liable to degenerate. This distinction of fewer oocytes arrested at the GV stage in high concentrations of CLZ was due

to the fact that those GV oocytes were degenerated and consequently classified as degenerated.

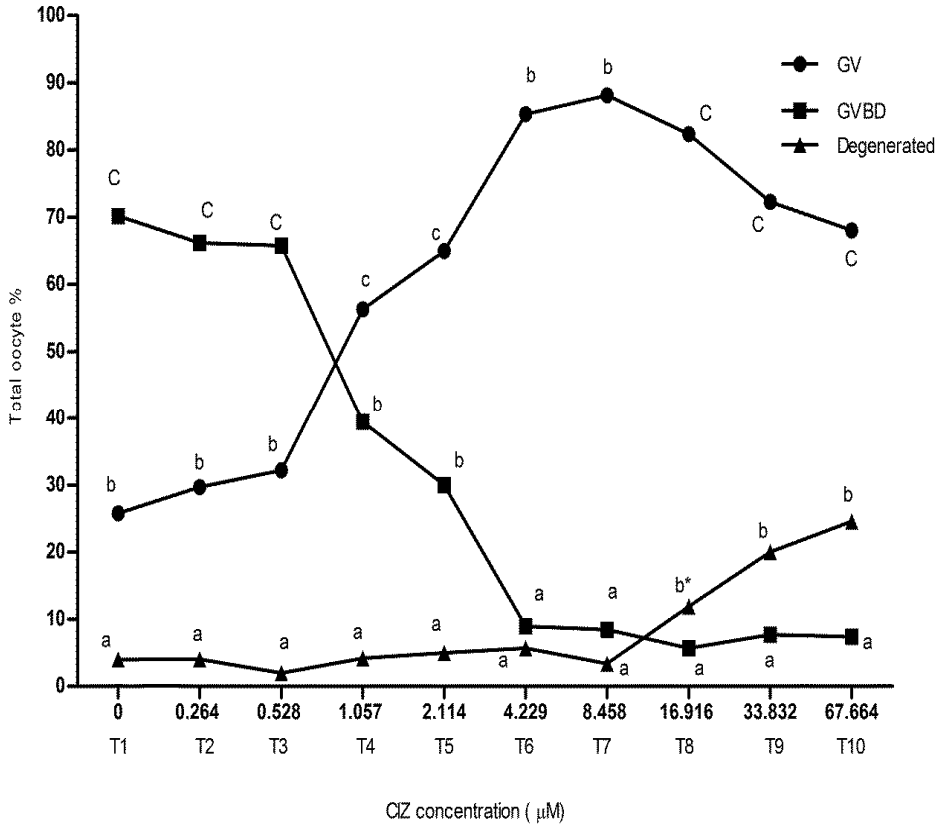

Figure 2.1. Cilostazol effects on oocyte meiotic maturation and morphology after 24h. Immature oocytes from antral follicles of hyperstimulated mice were denuded and incubated with 10 different CLZ concentrations and evaluated for their meiotic maturation and morphological normality after 24h. As CLZ concentrations were increased, more oocytes were arrested at germinal vesicle (GV) stage and less oocytes succeeded to undergo germinal vesicle breakdown (GVBD). At high CLZ concentrations, degeneration was noted targeting essentially the arrested oocytes at GV stage. T1, T2, T3, T4, T5, T6, T7, T8, T9, T10 were evaluated with numbers of oocyte corresponding to 450, 148, 152, 119, 100, 123, 118, 210, 130, and 122 oocytes. Data are presented as oocyte percent from the total number of scored oocytes at each treatment. Values with no common superscript within each treatment are significantly different at $p<0.0001$ except at b* and a superscripts with $P<0.05$ using Chi square test.

Oocytes that underwent germinal vesicle breakdown

A negative correlation was identified between increasing concentrations of CLZ and oocytes that underwent GVBD ($r=-0.88$, $P<0.0001$, Fig. 2.1). More oocytes were prevented from undergoing GVBD with CLZ concentrations of T4-10 ($P<0.0001$). The GVBD blocking effect was progressive along CLZ effective concentrations (T4-10) because T6-10 significantly reduced the GVBD oocytes more than T4-5 ($P<0.0001$, Fig. 2.1). Once CLZ was introduced to the control culture, the MII predominance over MI oocytes ($P<0.0001$, Fig. 2.2A) was lost. Moreover, concentrations of CLZ of T4-6 and T8-10 showed MI predominance over MII oocytes ($P<0.05$). A decline in MI oocytes along the different CLZ concentrations occurred at T6 through T10 in comparison to lower concentrations ($P<0.005$), while a significant reduction of the MII oocytes appeared at lower concentrations of T4 through T10 in comparison to control and T2-3 ($P<0.0001$). The effect of the MII oocyte reduction was progressive, as T6 through T10 exhibited less MII oocytes than T1-4 ($P<0.03$).

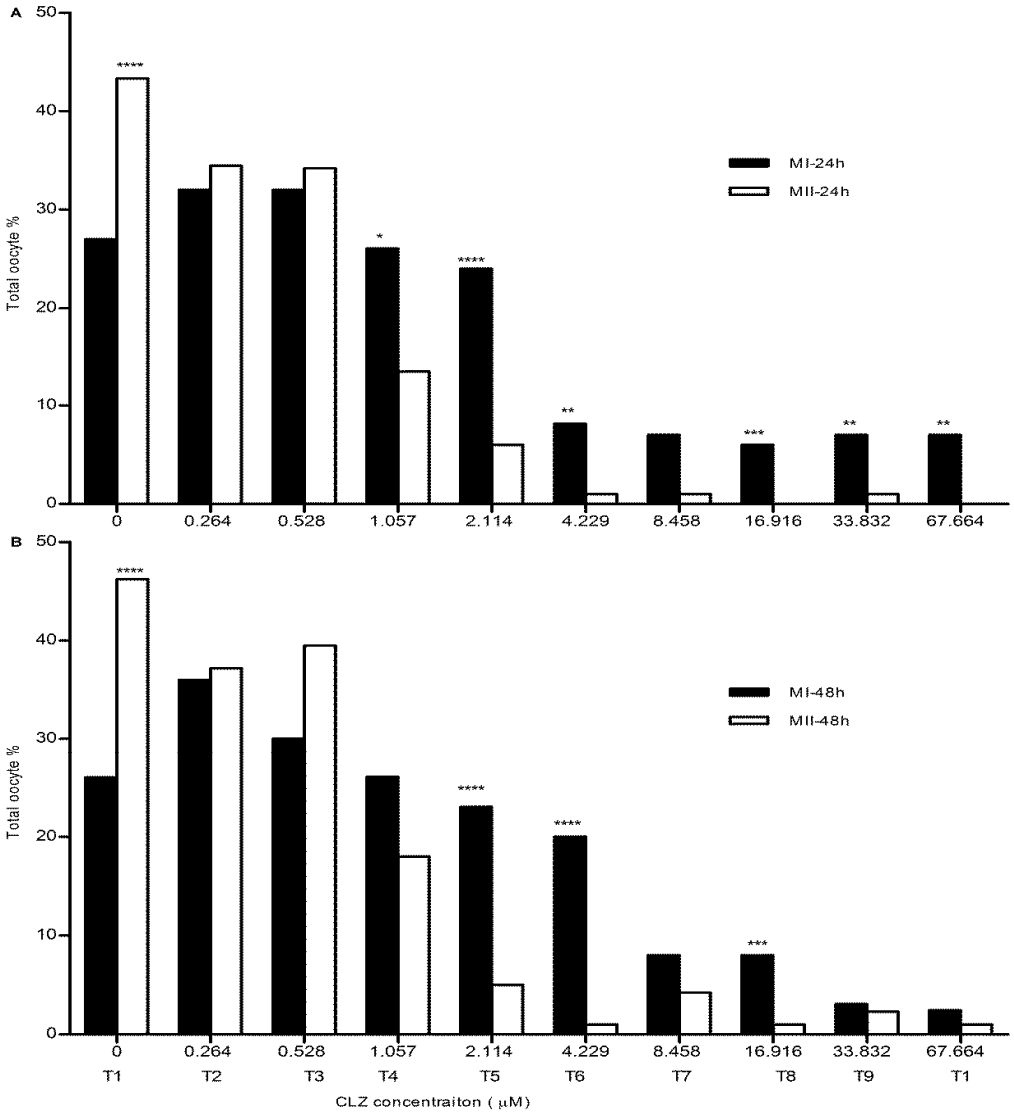

Figure 2.2. Metaphase I (MI) and metaphase II (MII) oocyte distribution after incubating immature oocytes with 10 different CLZ concentrations for 24h (A) and 48h (B). Once CLZ was introduced to the control medium, the significant predominance of MII over MI oocytes was lost. At the effective inhibitory concentrations of CLZ that start at T4, less MII oocytes were observed than MI oocytes. Data are presented as oocyte percent from the total number of scored oocytes of each treatment. Values with asterisk are significantly different within each treatment. *<0.05, **<0.01, ***<0.001, ****<0.00001 using Chi square test. T1, T2, T3, T4, T5, T6, T7, T8, T9, T10 were evaluated with numbers of oocyte corresponding to 450, 148, 152, 119, 100, 123, 118, 210, 130, and 122 oocytes.

Degenerated oocytes

The weakest correlation was observed between the different concentrations of CLZ and oocyte degeneration ($r=0.55$, $P<0.0001$, Fig. 2.1). After 24 h of incubation, CLZ concentrations of T9-10 showed higher numbers of degenerated oocytes when compared to T1-8 ($P<0.05$). The degeneration also elevated in T8 when compared to T1-4 and T7 ($P<0.01$ and $P<0.009$, respectively). Moreover, concentrations of T9-10 showed higher numbers of degenerated oocytes than oocytes with GVBD ($P<0.05$), but less than GV arrested oocytes ($P<0.0001$). This degenerative effect of the CLZ concentration of T8-10 was also noted when incubated oocytes were assessed as normal or abnormal based upon the morphological appearance of the oocytes (Fig. 2.3A), regardless of the stage of meiotic maturation ($P<0.01$). The overall distribution for vacuolation/fragmentation in degenerated oocytes revealed an increase in both fragmented and vacuolated type oocytes at the high CLZ concentrations of T9-10 ($P<0.05$) and T8-10 ($P<0.04$), respectively, compared to other concentrations. The overall trend for these 2 forms of morphologically degenerated oocytes showed that the vacuolated type oocytes were the predominant form of degenerated oocytes at the low and medium concentrations of CLZ, whereas both types of degenerated oocytes occurred at the high concentrations (Fig. 2.4).

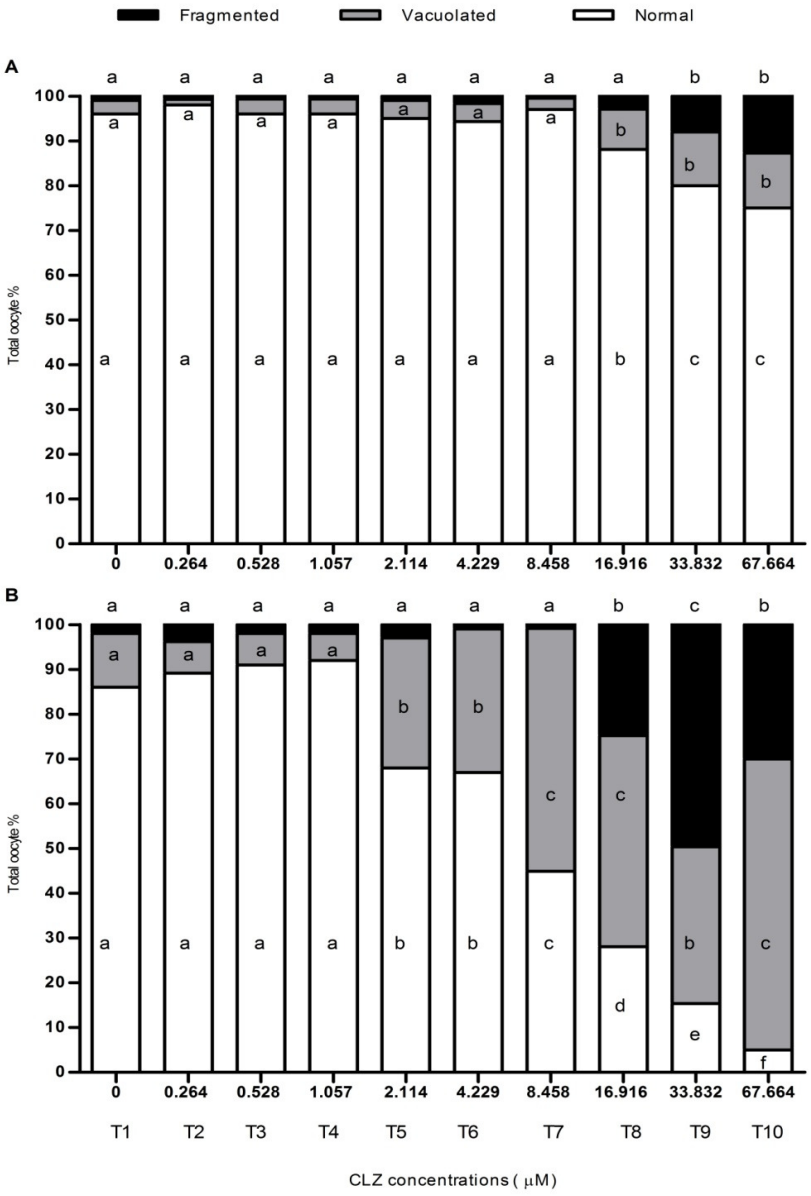

Figure 2.3. Degenerative impacts of different CLZ concentrations on oocyte morphology after 24h (A) and 48h (B). More degenerated oocytes were observed at higher CLZ concentrations than lower concentrations. Data are presented as oocyte percent from the total number of scored oocytes. Values with no common superscripts along the different treatments of the same oocyte type are significantly different ($P<0.05$).

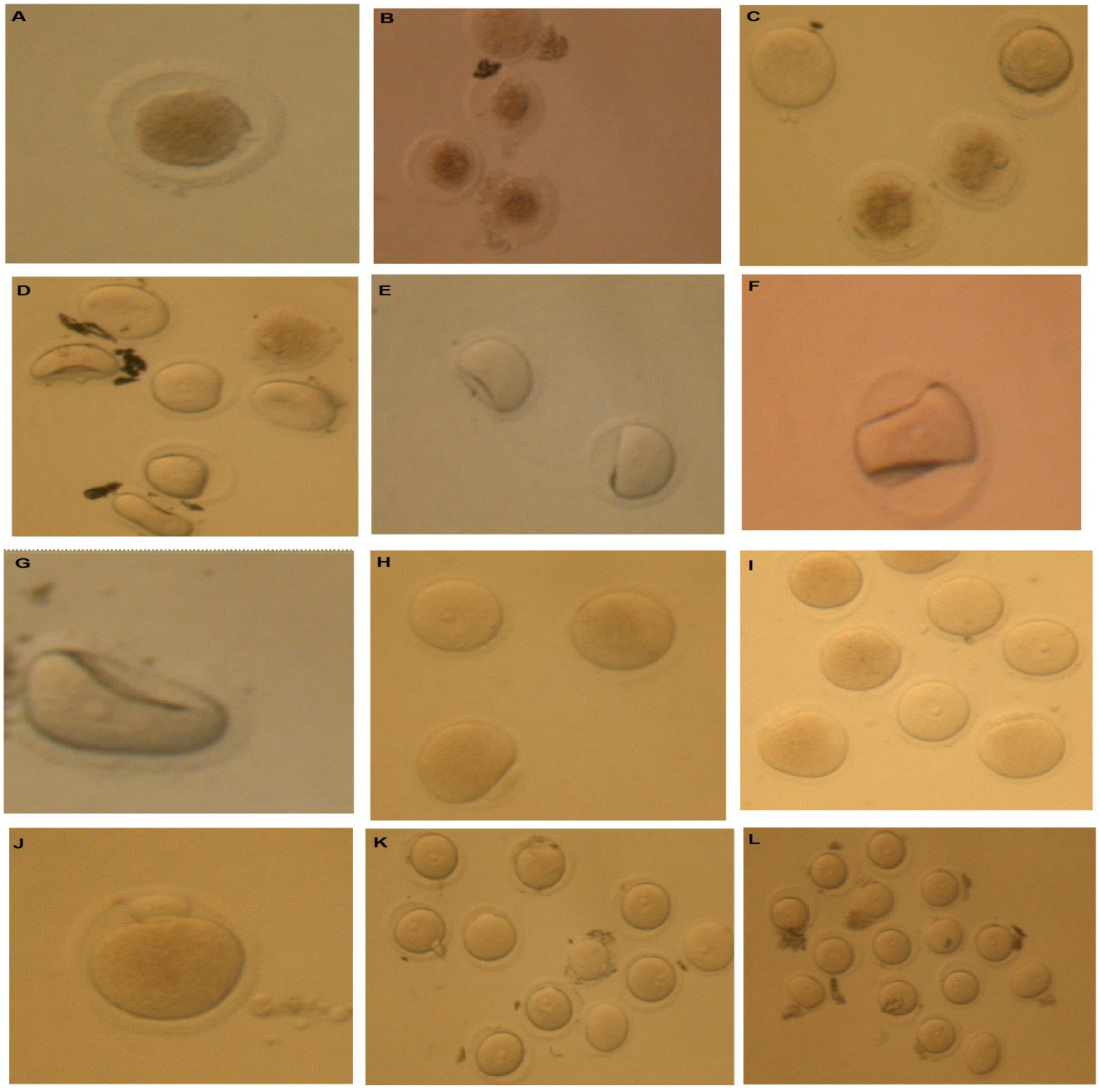

Figure 2.4. Degenerative impacts of different CLZ concentrations on oocyte morphology after 24h and 48h incubation. Fragmented degenerated oocytes displayed coarse granular, non-homogeneous, and dark (brownish-blackish) cytoplasm after incubating for 24 (A-B) and 48h (C) with16.916, 33.832, or 67.664 µM of CLZ, respectively. Vacuolated degenerated oocytes displayed non-spherical oocytes with or without a large perivitelline space after incubating for 24 (D-E) and 48h (F-G) with 8.458, 16.916, 33.832, or 67.664µM of CLZ, respectively. Morphologically normal oocytes evoked spherical shape, with smooth granular and transparent cytoplasm at different meiotic maturational stages after incubating for 24 (H-I) and 48h (J-L) with 0.264, 0.528, 0, 2.114, or 8.458µM of CLZ, respectively.

Forty eight hour incubation

Oocytes arrested at the germinal vesicle stage

No correlation was detected between the concentrations of CLZ and GV arrested oocytes (r=0.065, p=0.57, Fig. 2.5) due to a biphasic trend of increasing concentrations of CLZ on GV arrested oocytes (inverted U shape). This was further analyzed by examining the possible correlation among GV, GVBD, and degeneration outcomes which showed a strong negative influence of degeneration on GV oocytes (r=-0.938, P<0.0001) and to a lesser extent on GVBD oocytes (r=-0.822, P<0.0001). Interestingly, both GV and GVBD oocytes were positively correlated with each other (r=0.6, P<0.0001); two states that are usually inversely proportional for *in vitro* culture systems. CLZ at concentrations of T4-T7 was able to arrest more oocytes at the germinal vesicle stage when compared to other concentrations (P<0.0001). This effect was not continuous as T8 showed more GV arrested oocytes than T9 (19.52 *vs*. 10%, P=0.002) and T9 in turn was restraining more GV oocytes than T10 (10 *vs*. 1.6%, P=0.005). Collectively, these data indicate that the notable increase of degenerated oocytes was due to degeneration of GV and to less extent GVBD oocytes and consequently those oocytes were classifies as degenerated oocytes. This also means that the capability of CLZ to arrest oocyte meiotic maturation was constant up to 48 h and that it is the degeneration that was reducing the GV oocyte percentage.

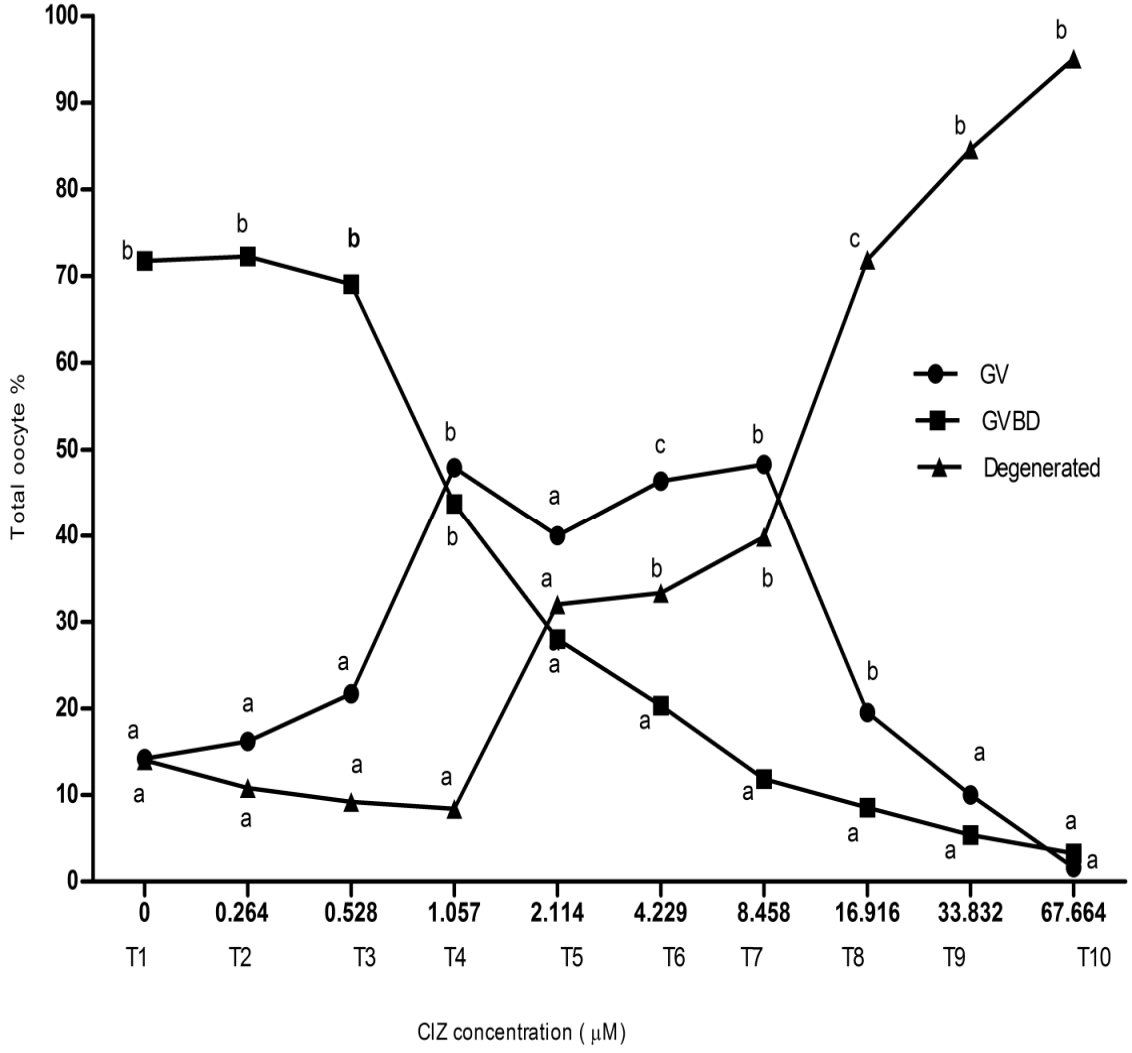

Figure 2.5. Cilostazol effects on oocyte meiotic maturation and morphology after 48h. Immature oocytes from hyperstimulated mice were denuded and incubated with 10 different CLZ concentrations and evaluated for their meiotic maturation and morphological normality after 48h. As CLZ concentrations were increased, the arrested oocytes at germinal vesicle GV stage started to decrease due to degeneration. CLZ shows a sustained inhibitory effect and prevented oocytes from undergoing germinal vesicle breakdown (GVBD) up to 48h. CLZ concentrations of T1, T2, T3, T4, T5, T6, T7, T8, T9, and T10 were evaluated with numbers of oocyte corresponding to 450, 148, 152, 119, 100, 123, 118, 210, 130, and 122 oocytes. Data are presented as oocyte percent from the total number of scored oocytes at each treatment. Values with no common superscript within each treatment are significantly different at $p<0.0001$ except at *b and a superscripts with $P<0.05$ using Chi square test.

Oocytes that underwent germinal vesicle breakdown

A negative linear correlation was observed between increasing CLZ concentrations and GVBD oocytes ($r=-0.927$, $P<0.0001$, Fig. 2.5). CLZ at concentrations of T4-T10 was able to reduce the spontaneous germinal vesicle breakdown when compared to the control group and to low CLZ concentrations of T2-3 ($P<0.0001$). Unlike the GV arresting effect of CLZ, the GVBD effect was continuous and as CLZ concentration increased, more oocytes were blocked from undergoing GVBD. The CLZ concentration of T6 prevented oocytes from undergoing GVBD in comparison to T4 ($P<0.0001$) while T10 prevented more oocytes from undergoing GVBD when compared with T7 ($P=0.012$). No comparative block was observed among T8, T9, and T10. Furthermore, scoring for GVBD oocytes revealed MI oocyte sovereignty from T4-10 in contrast to MII oocytes sovereignty in control and non-inhibitory concentrations ($P<0.0001$, Fig. 2.2B). Additionally, extending the incubation time from 24 to 48 h did not result in significant increases in oocytes undergoing GVBD or first polar body emission ($P>0.05$).

Degenerated oocytes

Linear correlation was also present between increasing concentrations of CLZ and the increase in degenerated oocytes ($r=0.874$, $P<0.0001$, Fig. 2.5). Increases in the number of degenerated oocytes after 48 h of CLZ exposure began at T5 when compared to T1-4 ($P<0.0001$, Fig. 2.3A and 2.5) whereas

scoring of the oocytes after 24 h of exposure showed T8 as the first concentration of CLZ to negatively impact oocytes (critical degenerative concentration). Furthermore, as the concentration of CLZ was increased beyond T5 for 48 h of exposure, the degenerative impact on oocytes was more vigorous. The CLZ concentration of T8 negatively impacted the morphology of oocytes when compared to T4-7 ($P<0.0001$), while treatments of T9 degenerated more oocytes than T8 ($P<0.01$) and T10 more than T9 ($P=0.006$).

When degenerative changes based on morphological assessment of normal or abnormal oocytes were analyzed without considering the meiotic status (Fig. 2.3B), we found that T5-10 decreased the number of normally appearing oocytes when compared to T1-T4 ($P<0.0001$), indicating a harmful effect of increasing CLZ concentrations on oocyte morphology. Moreover, each treatment within this degenerative range reduced the proportion of non-degenerated oocytes more than the previous treatment ($P<0.01$). As for fragmented and vacuolated degenerated oocytes (Fig. 2.3B), T5-10 showed more vacuolated degenerated oocytes when compared to control and T2-4 ($P<0.0001$). Also, T7-8 and T10 showed higher numbers of vacuolated oocytes than other concentrations ($P<0.0001$). Fragmented oocytes increased at the last three concentrations ($P<0.0001$), with a peak at T9 that exceeded the vacuolated type oocytes ($P<0.001$). Extending the incubation period from 24 h to 48 h did not change the expression of these degenerative forms as the vacuolated type oocytes were predominant from T1 through T8 ($p<0.01$), while

both degenerated forms were apparent at T8-10. Incubation up to 48 h with CLZ did not support further degenerative progression from vacuolation into fragmentation (Fig. 2.4).

Discussion

We show here for the first time that CLZ is able to block mouse oocyte meiotic maturation efficiently in an *in vitro* culture system at concentrations and efficiency that resemble other potent and well documented PDE3-I, such as cilostamide, Org 9935, and milrinone. It is hypothesized that inhibition of PDE3 by cilostamide is 10 times greater than that of CLZ, since the IC50 of cilostamide in platelets is 20nM and CLZ in vascular smooth muscle is 200nM (Bender and Beavo 2006; Tanaka *et al*. 1988). However, the present study has shown that 4.2 µM CLZ was able to arrest more than 85% of oocytes at the GV stage compared to a 100% arresting of oocytes at the GV stage by 1 or 5 µM cilostamide (Jee *et al*. 2009; Vanhoutte *et al*. 2008), or 89% of oocytes at the GV stage by 8.5 µM cilostamide (Coticchio *et al*. 2004) when mouse oocytes were treated for less than 24 h. It is worth noting that we used DO and not CEO, which were used in the above referenced studies. The information regarding the comparative efficiency between DO and CEO to undergo GVBD is contradictory. Mechanical isolation of the oocytes and denudation from other somatic follicular cells interrupt meiotic inhibitory communication between oocytes and follicular cells through the numerous gap junctions. Such interruption is believed to cut

the somatic compartment supply of inhibitory substances such as cAMP and cGMP, which are known to forestall the meiotic progression. Indeed gap junction interruption is believed to be a prerequisite to GVBD (Norris *et al*. 2008 and 2009; Sela-Abramovich *et al*. 2006 and 2008; Sherizly *et al*. 1988). Moreover, follicular oocytes can maintain the GV stage even after mechanical isolation from the ovaries if gap junctions are still intact and functional (Nogueira *et al*. 2005b). These data suggest that DO are free from the somatic meiotic inhibitory effects and are more liable to undergo GVBD than CEO. On the other hand, nuclear maturation rates were found to be higher when cumulus cells remained intact as opposed to being removed prior to *in vitro* maturation (Chang *et al*. 2005; Goud *et al*. 1998; Zhang *et al*. 1995). *In vitro* maturation of rat CEO shortened the time required for polar body emission by 2 h and increased the penetration rate and embryo competence more than DO matured *in vitro* (Vanderhyden and Armstrong 1989).

The proportion of DO shown to undergo GVBD was higher (while the GV proportion was lower) than those in CEO treated using the same PDE3-I (Nogueira *et al*. 2005b; Shu *et al*. 2008; Tsafriri *et al*. 1996). This suggests that DO were more likely to undergo GVBD than the enclosed type oocytes. Also, the overall preparation for these oocytes from the time of animal sacrifice until the beginning of incubation was approximately 15 minutes. Importantly, no oocyte meiotic maturational inhibitor was added to the culture medium during oocyte retrieval and preparation, demonstrating block of meiotic maturation was

due to CLZ. We suggest that these factors contributed to the lack of complete inhibition of meiotic maturation at the GVBD level, especially since no significant increase was observed in the number of MI and MII oocytes after 48h of incubation from that of 24h. This indicates that the initiation of GVBD may have already begun in some of the oocytes and that it was too late for CLZ to inhibit this process.

Interestingly, CLZ is able to maintain a constant inhibitory action on oocyte maturation, an effect not usually observed with other PDE3-Is. In this regard, Bilsdeau-Goeseels (2003) reported that trequinsin at concentrations of 10 and 50nM was unable to maintain its meiotic maturational inhibitory effect on bovine DO when the incubation period was extended from 7h to 22h. Furthermore, Shu *et al*. (2008) showed that 20μM cilostamide continuously lacked the inhibitory effect on GVBD of human CEO when meiosis was evaluated at different time points up to 48h. Conversely, the present study has showed a constant inhibitory effect of CLZ when the incubation period of mouse oocytes was prolonged from 24h to 48h.

The concentrations of CLZ evaluated in this study are relevant to humans since these levels have been observed clinically in both healthy people and those with intermittent claudication disease who are taking CLZ (Bramer *et al*. 1999). Single oral administration of 50, 100, or 200mg in normal healthy volunteers produced plasma concentrations of 411.03±83.5, 625.01±168, or 806.15±238μg/L, respectively, while the steady state plasma concentration after

100mg oral administration, twice daily, for 12 days was 1223±432µg/L. These plasma concentrations are similar to our CLZ concentrations of T4-T6 that showed a significant reduction in GVBD and an increase in GV arrested mouse oocytes. This suggests that doses of 50, 100, and 200mg CLZ, which are the normal dosages for treatment of intermittent claudication, may have contraceptive effects in woman if the compound is capable of inducing the same inhibitory effect on human oocyte maturation *in vivo*. At this time, however, there have been no reports of contraceptive effects in women who have taken CLZ therapeutically. It is possible, however, that most of the patients with intermittent claudication are past the normal reproductive age.

Oocyte degeneration was elevated as CLZ concentrations were increased. Knowing that the majority of these degenerated oocytes were able to be scored and were in the GV stage explains the reduction in the capability of arresting oocytes at the GV stage at high CLZ concentrations in comparison with lower concentrations. This lack of oocyte meiotic inhibition at high concentrations was not due to GVBD development but was due to GV oocyte degeneration. These observations indicate that only GV arrested oocytes, not GVBD oocytes, were degenerated at 24h. At 48h, we noted a greater degeneration on GV oocytes and less degeneration on GVBD. If our scoring system was only looking for the two end points of GV and GVBD, and not for degeneration as well (especially, we were able to score many of these degenerated oocytes), such classification would have shown that more than

92% of GV oocytes were arrested, instead of the lower percent noted when the CLZ treatment of 67.6 µM was evaluated. This decline in the percent of GV arrested oocytes can be designated as a pseudo-decline. These data indicate that CLZ is not losing its efficacy as a PDE3 inhibitor and it is stable during long term incubation.

An important point to be addressed whenever DO are cultured *in vitro* is the possibility of inducing compromised metabolic competence. Metabolic cooperativity and coupling is established between the oocytes and the follicular cells through delineated gap junctions (Heller *et al*. 1981; Pelland *et al*. 2009). Denuded oocytes are incapable of efficiently uptaking some essential nutrients, such as amino acids and adenosine triphosphate, from the culture medium when concentrations resemble those in plasma. The cumulus cells are thought to be controlled through oocyte paracrine factors to prompt them to uptake amino acids and supply the oocytes (Eppig *et al*. 2005). In this regard, specific amino acids and ribonucleosides are transferred into the oocyte through this metabolic coupling of cumulus cells (Colonna and Mangia 1983; Heller *et al*. 1981). Likewise, DO of optimum growth and developmental states could not continue 48h without degeneration under this condition of low nutrition. On the other hand, the marked degeneration noted in our study was proportional to the concentrations of CLZ and only the GV arrested oocytes were significantly sensitive during the 24h incubation to this degenerative impact.

Few data are available on oocyte degeneration and toxicity after direct chemical exposures. Research on oocyte development has mainly focused on understanding oocyte metabolic and biologic functions, as well as on assisted reproductive technologies such as cloning, *in vitro* maturation, and fertilization. Cellular toxicity can be attributed to oxidative stress such as plasma membrane lipid peroxidation, immunological inflammation such as cytokine over release, cytogenetic damage such as DNA strand fragmentation, or others. Tamura *et al*. (2008) reported that human degenerated oocytes have high concentrations of 8-hydroxy-2`-deoxyguanosine (a biomarker for oxidative stress), and that the addition of hydrogen peroxide significantly reduced the number of *in vitro* matured oocytes in a dose dependent manner. Interestingly, CLZ concentrations of 30-100µM similar to our tested concentrations have been shown to inhibit oxidative stress–induced premature senescence via activation of endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells (Ota *et al*. 2008). On the other hand, eNOS knock-out mice showed more oocytes with atresia-related apoptosis relative to those in wild type mice (Jablonka-Shariff and Olson 1998). Human neuroblastoma and brain microvascular endothelial cells were found to augment metallothionein (MT), scavengers of reactive oxygen species (ROS), after exposure to 1-50µM CLZ *in vitro* (Suzuki *et al*. 2007). Furthermore, mRNA expression of antioxidant enzymes such as reduced glutathione (GSH), g-glutamyl-cysteine transferase (GCS), and superoxide dismutase (SOD) in mouse, bovine, and human oocytes have been confirmed

(El Mouatassim *et al*. 1999; Lonergan *et al*. 2003; Sugino 2005). GSH was shown to be proportionally correlated with fertilization rate and embryonic development, but did not affect the progression of meiotic maturation (De Matos *et al*. 2002 and 2003; Gasparrini *et al*. 2006; Zhou *et al*. 2010). With regard to CLZ, the compound restored GSH level in diabetic human and rat nephropathy, which protected against a pathological inflammatory burden produced by hyperglycemia induced-ROS (Lee *et al*. 2010). In regard to immunological inflammatory effects, the compound inhibited bacterial lipopolysaccharide (LPS) stimulated pro-inflammatory cytokine production in macrophages (at 30 and 40μM) and rescued the mice from LPS-induced inflammation and mortality (Park *et al*. 2010).

Although temporary nuclear arrest of human GV oocytes with 1μM of cilostamide for 24h produced more oocytes with normal bipolar spindles and aligned chromosomes than non-arrested oocytes (Vanhoutte *et al*. 2007), the *in vitro* matured oocytes in mice showed significantly greater DNA fragmentation than *in vivo*-matured oocytes (Huang *et al*. 2008). The magnitude of DNA damage (comet tail length) was greater for *in vitro* matured bovine oocytes than those treated with antioxidants (Mukherjee *et al*. 2010). Shin *et al*. (2004) showed that CLZ concentrations of 0.01-100μM inhibited DNA fragmentation and apoptosis of human umbilical vein endothelial cells cultured with remnant lipoprotein particles for 24 h. Hong *et al*. (2003 and 2006) showed a preventive effect of 0.1-10μM CLZ on the prominent DNA fragmentation of human brain

neuroblastoma cells treated with TNF-α for 24h. Lee *et al*. (2004) confirmed this neuroprotective effect of CLZ when the compound was administered orally to rats induced with cerebral ischemic injury, and demonstrated that the affected neurons showed a suppressed DNA fragmentation laddering feature and low apoptosis.

Collectively, these data suggest that CLZ has cellular protective properties against ROS, inflammation, and DNA fragmentation. CLZ also restores the levels of MT, GSH, and eNOS. Moreover, relating oocyte degeneration to a lack of metabolic coupling upon denudation from other cells is not supported by our observation of a positive correlation between CLZ concentration and essential targeting of GV arrested oocytes. This information, along with our results, leads us to suggest that prolonged sustaining of oocytes at the GV stage is harmful. Persistent and cumulative high levels of cAMP could be responsible for this degenerative effect, as a majority of these degenerated oocytes were from the GV arrested oocyte pool. A continuously high intraoocyte cAMP level is suggested to be negatively correlated to oocyte quality and normality. The potency and duration of PDE3 inhibition and cAMP accumulation could be the reason for the contradictory results related to the beneficial effect of *in vitro* meiotic synchronization on oocyte competence, *in vitro* fertilization, and embryo development (Curnow *et al*. 2011; Jee *et al.* 2009). Further studies are required to gain insight as to the effects of cAMP levels on oocyte viability, competence, and developmental capacities. Also, potential toxic effects of CLZ

on oocytes should be addressed. Studies are also needed to further understand the reason(s) of vacuolation and fragmentation of oocytes caused by different concentrations of the CLZ.

Summary and conclusion

- Cilostazol inhibits spontaneous oocyte maturation *in vitro* in mice.
- The inhibitory concentrations of CLZ on oocyte maturation are similar to those found in healthy humans who were reported in clinical trials.
- Prolonged arrest of oocyte maturation at the germinal vesicle stage is harmful and the arrested oocytes are undergoing degeneration.
- Sustained high level and/or accumulation of cAMP in the arrested oocytes may explain the noted oocyte degeneration.
- Degenerated immature oocytes have 2 distinctive patterns; vacuolated and fragmented.

CHAPTER III

CILOSTAZOL INHIBITS OOCYTE MEIOTIC MATURATION IN SUPEROVULATED MICE

Overview

Previously, we evaluated the inhibitory effects of CLZ on oocyte maturation. We found CLZ is able to block oocyte maturation *in vitro*. Here, we investigated the influence of time of administration of CLZ on oocyte meiotic maturation *in vivo* and defined protocols for the collection of ovulated oocytes of different meiotic stages. Mice were superovulated and gavaged with 0, 7.5, or 15mg CLZ, once or twice, at various times around human chorionic gonadotropin (hCG) injection. Ovulated oocytes were collected at different time points and analyzed for maturation and cAMP levels. CLZ resulted in the ovulation of metaphase I oocytes after a single dose of CLZ as early as 9h pre-hCG. This inhibitory effect was greater when CLZ administration was closer to the hCG injection time with an increase in germinal vesicle oocyte yield, especially with two doses of CLZ. The inhibitory effect of CLZ disappeared when administered 4h post-hCG. Ovulated GV oocytes had low cAMP levels in comparison to ovarian GV oocytes, which may be attributed to gap junction interruption and/or CLZ plasma concentration reduction. It is concluded that CLZ inhibits oocyte meiosis with a wide range of times of administration, which accounts for its contraceptive properties. Acquisition of oocyte meiotic competence in the oviduct upon late

collection indicates CLZ reversibility and can be utilized to synchronize oocyte meiotic and cytoplasmic maturation in superovulation. Finally, protocols for ovulating meiotically immature oocytes are presented that can substitute for conventional ovarian immature oocyte recovery.

Introduction

The capability of the ovarian oocyte to undergo spontaneous oocyte meiotic maturation *in vitro* was first observed by Pincus and Enzmann in 1935. An inhibitory role for cAMP on spontaneous maturation was suggested in 1974 by Cho *et al.* after observing *in vitro* inhibition of oocyte meiotic maturation caused by dibutyryl cAMP and theophylline. In 1983, Vivarelli *et al.* observed the inverse relationship between cAMP concentration and oocyte meiotic maturation. Cyclic-AMP synthesis by the somatic cells surrounding oocytes was confirmed the same year, further supporting the inhibitory role of cAMP on oocyte meiotic maturation. Moreover in 1983, Schultz *et al.* demonstrated oocyte meiotic maturation inhibition by the three cAMP augmenting loops: adenylate cyclase activation, PDE inhibition, and cAMP analogues. They also presented the first evidence of cAMP regulating oocyte meiotic maturation *in vivo*. One year later, oocyte PDE was found to play a detrimental role in meiotic competence through controlling oocyte cAMP hydrolysis (Bornslaeger *et al*. 1984). In 1985, other cAMP related molecules such as adenosine and hypoxanthine were detected in oocyte follicular fluid (Eppig *et al*. 1985). In later decades, countless studies

were conducted and PDE-Is were found to prevent bovine (Thomas and Armstrong 2002; Thomas *et al*. 2004), porcine (Grupen *et al*, 2006; Miyano *et al*. 1995), rodent (Nogueira *et al*. 2003a; Tsafriri *et al*. 1996; Wiersma *et al*. 1998), monkey (Curnow *et al*. 2011; Jensen *et al*. 2002), and human (Nogueira *et al*. 2003b; Shu *et al*. 2008; Vanhoutte et al. 2007) oocyte meiotic maturation *in vitro*. Those PDE-Is were also able to arrest oocyte meiotic maturation *in vivo* in rodents (Wiersma *et al*. 1998) and monkeys (Jeffrey *et al*. 2010; Jensen *et al*. 2005 and 2008) and to block pregnancy in rodents (Wiersma *et al*. 1998) and monkeys (Jeffrey *et al*. 2010).

Phosphodiesterase 3A is the predominant PDE3 isoform in oocytes. The gain of meiotic competence was observed after peak PDE3A activity, indicating a pivotal prerequisite role for increasing PDE3A activity prior to meiotic competence. On the other hand, oocytes deficient in PDE3A were found to have a sustained cAMP level and failed to undergo *in vitro* maturation (Masciarelli *et al*. 2004; Richard *et al*. 2001; Sasseville *et al*. 2006). In contrast to intra-oocyte cAMP, the surrounding somatic cell cAMP was reported to play the opposite role in meiotic maturation. Increasing follicular cell cAMP is achieved via PDE4 inhibition and was found to induce *in vitro* meiotic maturation of follicular oocytes. Oocytes enclosed within follicles and incubated with rolipram (PDE4 inhibitor) underwent GVBD; however, spontaneous meiotic maturation of denuded oocytes was not arrested by rolipram but was arrested by PDE3-I. In addition to the opposite roles of somatic and germinal cAMP, PDE3 and PDE4

were found to be compartmentally localized in oocytes and granulosa cells, respectively (Tsafriri *et al*. 1996). Interestingly, PDE4 is believed to associate with other reproductive endocrinological events. In this regard, mice which lacked PDE4 showed a defect in ovulation and impaired fertility, whereas administration of a PDE4 inhibitor in FSH-primed rats resulted in ovulation of normal oocytes, indicating that PDE4 inhibitors can substitute for hCG in superovulation protocols (Conti 2011; Mayes and Sirard 2002; McKenna *et al*. 2005; Park *et al*. 2003).

The surge of LH, in addition to ovulation induction, triggers oocyte meiotic resumption. While many studies suggest different molecular mechanisms by which LH induces meiotic maturation of the follicular oocytes, the gap junction communication (GJC) disruption theory is the most common understanding. Interruption of communication between the somatic and germ cells has been confirmed as a prerequisite for meiotic maturation. Luteinizing hormone was observed to increase follicular cell production of cAMP and to block the numerous gap junctions in mural granulosa and cumulus cells via mitogen-activated protein kinase activation (Hashimoto *et al*. 1988; Isobe *et al*. 1998; Norris *et al*. 2008). Importantly, the administration of a gap junctional blocker has been shown to arrest maturation of follicular oocytes *in vitro* and *in vivo* (Sela-Abramovich *et al*. 2006 and 2008). Active gap junctions were found to provide oocytes with meiotic inhibitory substances such as cGMP. A dramatic loss of junction net area and interactions are indeed correlated with meiotic competence

(Larsen *et al*. 1986; Norris *et al*. 2009). High intraoocyte cAMP concentrations build the free catalytic subunit of protein kinase A (PKA) leading to deactivation of the M phase promoting factor and arrest of meiotic maturation (Han and Conti 2006; Oh *et al*. 2010).

In this study, we studied CLZ and tested its ability to reduce oocyte maturation *in vivo* using mice. We also defined the range of times of administration at which CLZ can start and lose its inhibitory effects in superovulated mice. We further examined the capability of obtaining ovulated oocytes that are meiotically immature; GV and/or MI, depending upon the dose, time, and frequency of CLZ administration. We then monitored the capability of the ovulated immature oocytes to initiate and to continue the meiotic maturation process in the oviduct to determine if we can have an additional method to obtain immature oocytes and to aid synchronization of oocyte meiotic maturation *in vivo* during superovulation.

Materials and methods

Animals

Swiss Webster mice (8-10 weeks old) were purchased from Harlan Laboratories (Houston, TX). Mice were housed in the Texas A&M University Laboratory Animal Facility in groups of 5-8 mice per cage under controlled temperature (23℃) and light/dark (14/10 h) cycle. Food and water were provided *ad libitum*.

All experiments and procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Chemicals and reagents

Pregnant mare serum gonadotropin (Folligon®) and hCG (Chorulon®) were purchased from Intervet Inc (Summit, NJ). Leibovitz's L-15 medium was purchased from Invitrogen (Grand Island, NY). Human tissue fallopian (HTF) medium with HEPES for murine IVF was obtained from Zenith Biotech (Guilford, CT). Bovine hyaluronidase and DMSO were purchased from Sigma (St. Louis, MO). CLZ was purchased from LKT Laboratories (St. Paul, MN).

Cilostazol dose determination

Cilostazol volume of distribution (V_d), as determined in humans, was used to determine the dosage of CLZ for the mouse. In humans, a non-compartmental model showed an apparent V_d of 2.76L/kg suggesting an extensive tissue distribution (Bramer *et al*. 1999), whereas a two-compartmental model suggested volumes of central and peripheral compartments of 20.5 and 73.1L, respectively (Yoo *et al*. 2010). The later model had a better fit than the one-compartment model when V_d was considered for mice, as shown by our extrapolated doses. Previously, we determined the *in vitro* inhibitory CLZ concentrations on denuded oocyte meiotic maturation in mice (Chapter II). The

inhibitory CLZ concentration was based on CLZ plasma concentrations of people who are taking cilostazol. Starting from the highest *in vitro* inhibitory concentrations (relevant to the reported highest human CLZ plasma concentration) as the desired initial mouse plasma concentration and the reported human central V_d, the 7.5 and 15mg doses, used in these experiments, were determined and were found to be effective in arresting oocyte meiotic maturation *in vivo*.

Experimental design

These experiments were designed to test the following hypotheses: 1) CLZ inhibits oocyte meiotic maturation in superovulated mice; 2) The effect of CLZ on oocyte meiotic maturation is reversible; 3) The doses and times of administration of CLZ with respect to hCG injection influence the meiotic maturation stage of the ovulated oocytes; 4) Immature oocytes are able to resume meiotic maturation in the ovary and/or oviduct at various times after CLZ administration. Fig. 3.1 shows the design of the experiments that were conducted to test these hypotheses.

All animals were injected intraperitoneally, at 6:00pm, with 7.5IU PMSG and after 47h with 7.5IU hCG. CLZ doses of 7.5 or 15mg were dissolved in 0.1 or 0.15ml DMSO, respectively, and administered fresh to mice via gastric gavage at 9, 7, or 4h pre-hCG, at the same time as hCG, or 2 or 4h post-hCG.

Some animals were treated twice with 7.5mg CLZ at 4h pre-hCG and 2h post-hCG and others at the same time as hCG and 6h post-hCG.

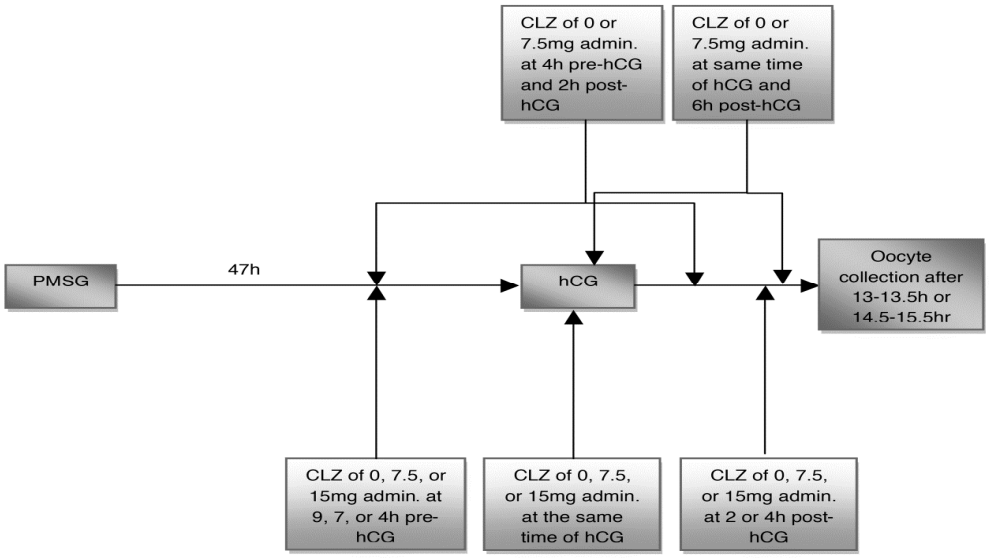

Figure 3.1. The experimental design of oocyte collection at different times post-hCG from mice treated with CLZ of different doses and at different times of administration.

Oocyte retrieval

Anesthetized animals were sacrificed by cervical dislocation and oocytes were collected 13-13.5h (early collection) or 14.5-15.5h (late collection) post-hCG injection. Ovaries and oviducts were excised and placed into 2ml of L-15 medium supplemented with 10% BSA and 4.2μM CLZ to prevent spontaneous oocyte meiotic maturation (Chapter II). The visibly distended region of the oviductal ampulla was punctured, and the ovulated CEO complexes were

collected and transferred to 1ml of 0.006% bovine hyaluronidase (dissolved in L-15 medium plus 10% BSA and of 4.2μM CLZ) for less than 1.5 minutes with occasional pipetting. Denuded oocytes were transferred again to 1ml medium similar to the initial medium and scored for maturational status as GV, MI, or MII (Wassarman *et al*. 1976) using a Nikon SMZ1500 stereomicroscope and Olympus IX71 inverted microscope. Control animals received the designated DMSO volumes at the same time as CLZ, and oocytes were collected at the same time as the treated animals to evaluate their oocyte meiotic maturation. The entire technique was conducted on a warm stage set at 38℃.

Determination of GVBD timing in superovulated mice

Non-treated female mice were superovulated as described in the previous section and oocytes were collected at 3, 6, or 9h post-hCG. The ovaries were excised in 1ml HTF medium supplemented with 10% BSA and 4.2μM CLZ. Oocytes were isolated as described previously (Wiersma *et al*. 1998) and assessed for their maturational stage.

Cyclic AMP assay

Cyclic AMP concentrations were measured in ovulated GV oocytes retrieved from animals treated with 7.5mg CLZ administered at the same time as hCG and 6h post-hCG, ovulated MI oocytes retrieved from animals treated with 7.5mg CLZ at 4h pre-hCG, and ovulated MII oocytes retrieved from non-treated mice

(negative control). Ovarian denuded GV oocytes from PMSG-primed mice were collected after 47h and used as positive controls. Pools of 40-65 oocytes per sample were used to assess cAMP contents. However, for the ovulated oocytes, the levels of cAMP were below the limit of the assay. Therefore, in some subsequent experiments, pools of 140-150 ovulated oocytes were analyzed (4-6 replicates). Briefly, ovarian GV or ovulated GV, MI, or MII oocytes were collected in HTF medium with 4.2μM CLZ supplemented with 10% BSA. Oocytes were denuded from cumulus cells, washed, and placed in 125μl of 0.1M HCl for 10 minutes at room temperature with occasional vortexing. The samples were centrifuged for 10 minutes at 800g. Supernatants were kept at -20℃ until analyses by an ELISA cAMP kit from Biomol Research Laboratories (Farmingdale, NY, USA) using an acetylating protocol as described in the manufacturer's instructions.

Statistics

A Chi square test was used to evaluate the differences between CLZ-treated and control animals and between the 7.5 and 15mg CLZ-treated animals for similar oocyte maturation stage of the same treatment time-point. The Kruskal-Wallis test followed by the Mann-Whitney U *post hoc* test was used to compare differences within each treatment time point and to compare differences of each oocyte maturational stage among different treatment time groups for each tested dose. The same analysis was used to test the differences in proportions of

GVBD oocytes in superovulated non-treated mice. Data from early and late oocyte collections of each cell-type were subjected to arcsine square-root transformation to normalize data before Student T-test was used. The Student T-test analysis was also used to analyze the difference between cAMP concentrations of ovulated and ovarian GV oocytes. The threshold for significant difference was set at $P<0.05$, and SPSS® 14.0 software (SPSS Inc., Chicago, IL) was used to carry out the statistical analyses.

Results

Single CLZ dose inhibited oocyte meiotic maturation

Early collection of oocytes from animals treated orally with single CLZ doses of 7.5 or 15mg, at the indicated times except for the 4h post-hCG time point, inhibited oocyte meiotic maturation at MI or at GV and MI, significantly ($p<0.0001$), when compared to relevant oocyte types from control animals in which more than 97% of ovulated oocytes were at the MII stage (Fig. 3.2 and 3.3). The overall trend of ovulated oocytes in animals treated with a single CLZ dose of 7.5mg showed a higher distribution for MI oocyte than other cell types

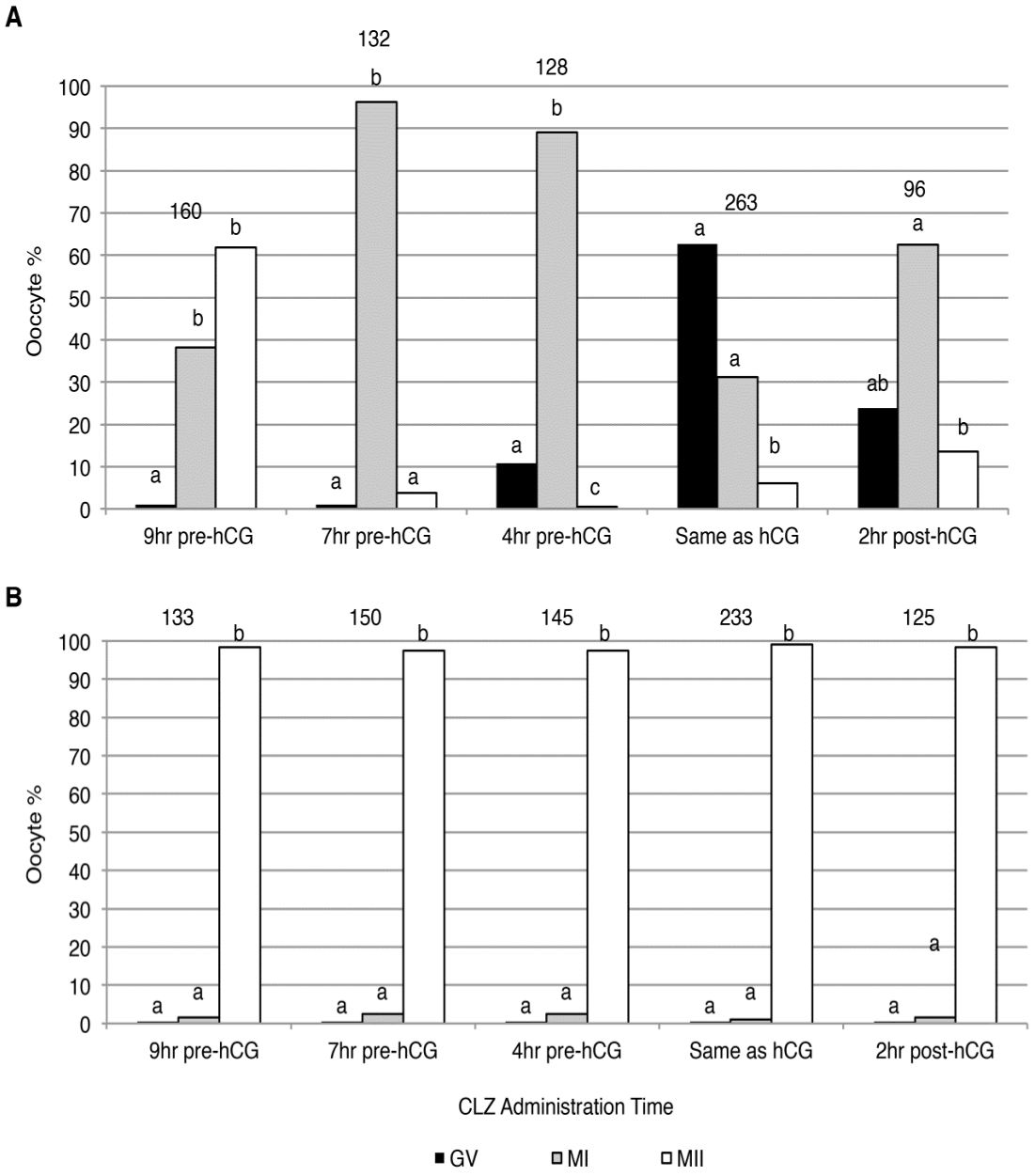

Figure 3.2. Cilostazol (7.5mg) effects on oocyte meiotic maturation in superovulated mice. Animals were superovulated and treated with CLZ (7.5mg/0.1ml DMSO) at the indicated times and ovulated oocytes were scored for meiotic status 13.00-13.5h post-hCG (A). Animals were superovulated and treated with 0.1ml DMSO (vehicle control) at the corresponding timings and ovulated oocytes were scored for meiotic status 13.00-13.5h post-hCG (B). Number at the top of each administration time is the total number of scored oocytes. Data are presented as oocyte percent from the total number of oocytes. Values with no common superscript within each treatment time are significantly different (p<0.05) using the Mann-Whitney test.

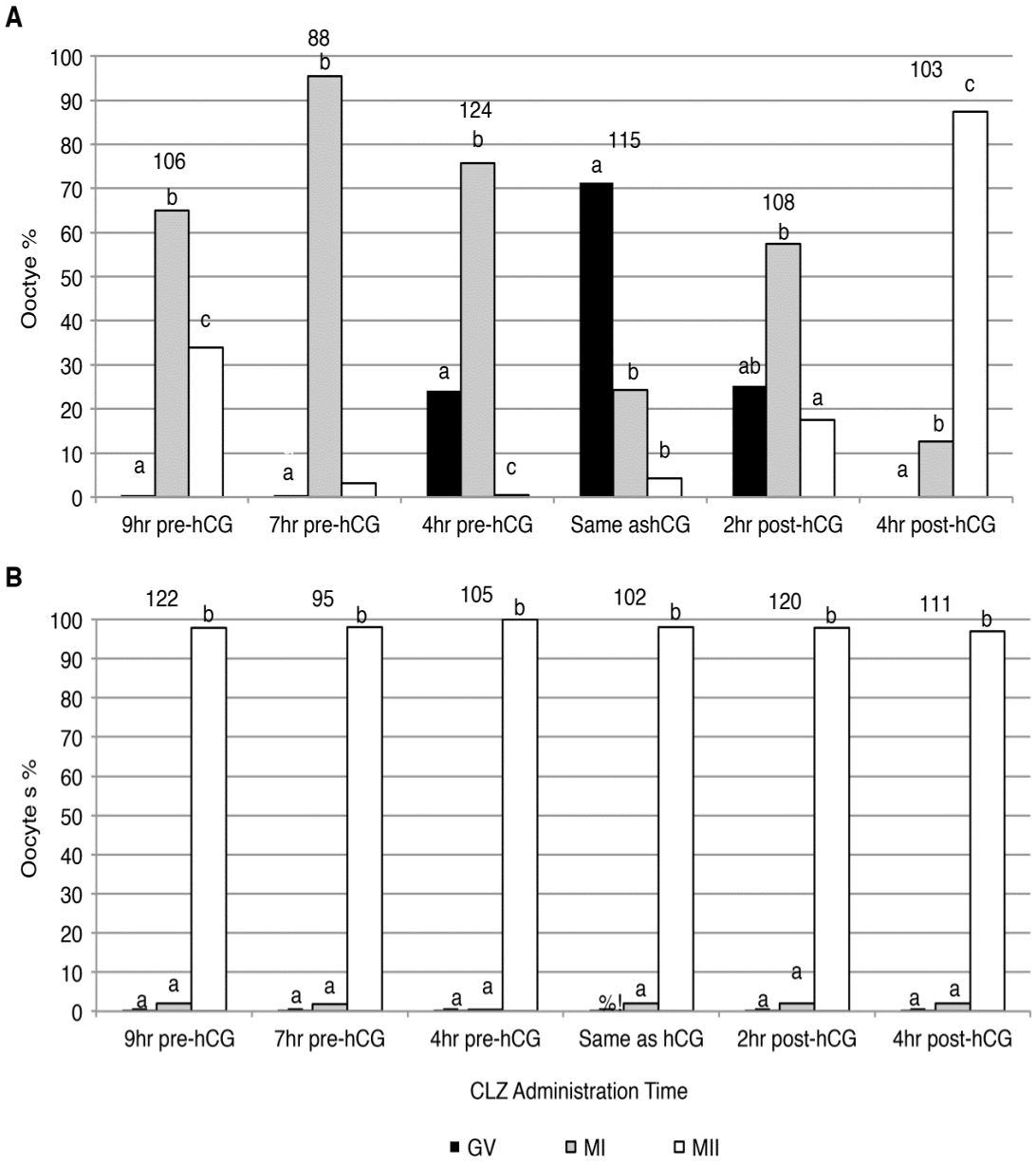

Figure 3.3. Cilostazol (15mg) effects on oocyte meiotic maturation in superovulated mice. Animals were superovulated and treated with CLZ (15mg/0.15ml DMSO) at the indicated times and ovulated oocytes were scored for meiotic status 13.00-13.5h post-hCG (A). Animals were superovulated and treated with 0.15ml DMSO (vehicle control) at the corresponding timings and ovulated oocytes were scored for meiotic status 13.00-13.5h post-hCG (B). Number at the top of each administration time is the total number of oocyte scored. Data are presented as oocyte percent from the total number of oocytes. Values with no common superscript within each treatment time are significantly different (p<0.05) using the Mann-Whitney test.

(Fig. 3.2A) at all tested time points except for two times of administration; 9h pre-hCG and same as hCG. The 9h pre-hCG administration allowed mice to ovulate more MII than MI oocytes (61.8% *vs*. 38.12%, P=0.1). Administration of CLZ at the time of hCG showed that GV oocytes were the predominant cell-type over the MI and MII oocytes (62.73% *vs*. 31.17% and 6%, P=0.1 and P<0.0001, respectively). The predominant GV oocyte ovulation with single CLZ dose administered at the time of hCG injection could be attributed to sufficient inhibitory CLZ blood concentration to block the early events of oocyte GVBD and thus delay the resumption of maturation. The characteristic MI oocyte ovulation in the single dose of 7.5mg CLZ treated animals was more marked in the groups treated at 7 and 4h pre-hCG than other times (P<0.01, Fig. 3.2A). Metaphase I oocyte ovulation prevalence continued with the 15mg CLZ dose (Fig. 3.3A) and was reduced only when CLZ was administered at the time of hCG due to increased GV oocyte ovulation over MI and MII oocytes (71.3% *vs*. 24.34% and 4.3%, respectively, P<0.04 for both). This could be due to the sufficient inhibitory concentration of CLZ in the circulation mentioned previously. The ovulation of MI oocytes also decreased at the 4h post-hCG time point due to increased ovulation of MII over MI oocytes (87.37% *vs*. 12.62%, P=0.003, Fig. 3.3A). Neither MI nor MII oocyte yield between CLZ and DMSO treatments at 4h post-hCG differ significantly (P>0.05), indicating a lack of CLZ arresting capability at this time point (Fig. 3.3). This administration time point (4h post-hCG) represents a cutoff at which PDE3-I is ineffective in impeding oocyte

meiotic maturation as the LH like activity of the hCG hormone is assumed to trigger meiosis events shortly after its administration.

Although administration of 7.5mg CLZ, 9h pre-hCG, resulted in more MII than MI ovulated oocytes (61.8% *vs*. 38.12%, P=0.1, Fig. 3.2A), the administration of 15mg at this time point reversed the effect and resulted in mice ovulating more MI oocytes than MII (65% *vs*. 33.9%, respectively, P=0.006, Fig. 3.3A), which is an evidence of a dose response effect. Also, the 15mg administration at this time-point resulted in more MI and fewer MII oocytes than did 7.5mg (P=0.0001, for both cell types). Animals treated with 7.5 or 15mg CLZ at 7h pre-hCG, ovulated mainly MI oocytes (>95%) with less than 4% MII oocytes (P<0.004), whereas animals treated 4h pre-hCG with 7.5 or 15mg CLZ ovulated only MI (>75%) and GV (<24%) oocytes with a significant difference (P<0.003) between the only two ovulated oocyte types. The 4h pre-hCG time treatment of 7.5 or 15mg CLZ resulted in a complete immature oocyte ovulation at combined GV and MI levels with no MII ovulation (P<0.05) in comparison to other times of administration. Moreover, comparing 7.5 to 15mg CLZ at 4h pre-hCG resulted in a significant increase of GV oocytes (10.8% *vs*. 24.1%, P=0.005, respectively) and decrease of MI oocyte ovulation (89.1% *vs*. 75.8%, P=0.005, respectively), demonstrating another dose response effect. Both doses of 7.5 or 15mg administered at 7h or to a lesser extent at 4h pre-hCG were sufficient to synchronize the ovarian oocyte GVBD and to uniformly ovulate a majority of MI oocytes (Fig. 3.2A and 3.3A). The ovulated oocytes at GV stage

started to increase notably (P<0.005) first in the single CLZ treatments (7.5 or 15mg) at the 4h pre-hCG time-point. This trend continued with CLZ administration at hCG or 2h post-hCG timing, with the highest GV oocyte yield at hCG injection. The GV oocyte rates from the GV ovulating protocols were not significantly different (Fig. 3.2A and 3.3A). The mixed ovulation of GV, MI, and MII oocytes started when CLZ of 7.5 or 15mg was administered 2h post-hCG with marked MI oocyte ovulation over MII oocytes (P<0.04). Figure 3.4 shows oocytes of different meiotic developmental capacity at early collection of oocytes from female mice treated with different CLZ protocols.

Multiple CLZ doses inhibited oocyte maturation at GV stage

Administration of 7.5mg CLZ, BID; 4h pre-hCG and 2h post-hCG or at the same time as hCG and 6h post-hCG, resulted in mice ovulating GV oocytes in comparison to lack of GV oocytes in their relevant control animals (P<0.0001, for both protocols, Fig. 3.5A). The ovulated GV oocytes in both protocols were greater than their corresponding MI oocytes (89.7% and 96% *vs*. 10.2% and 4%, respectively, P<0.003). The ovulated MI oocytes in those mice were significantly greater than the ovulated MII oocytes (P<0.03, for both protocols). The two protocols did not significantly differ in GV oocyte arresting capability at early collections (P=0.2) but resulted in the ovulation of significantly more GV oocytes than the single CLZ dose regimens (P<0.0001). Figures 3.5B and C show the

effect of multiple administrations of CLZ on oocyte meiotic maturation in superovulated mice.

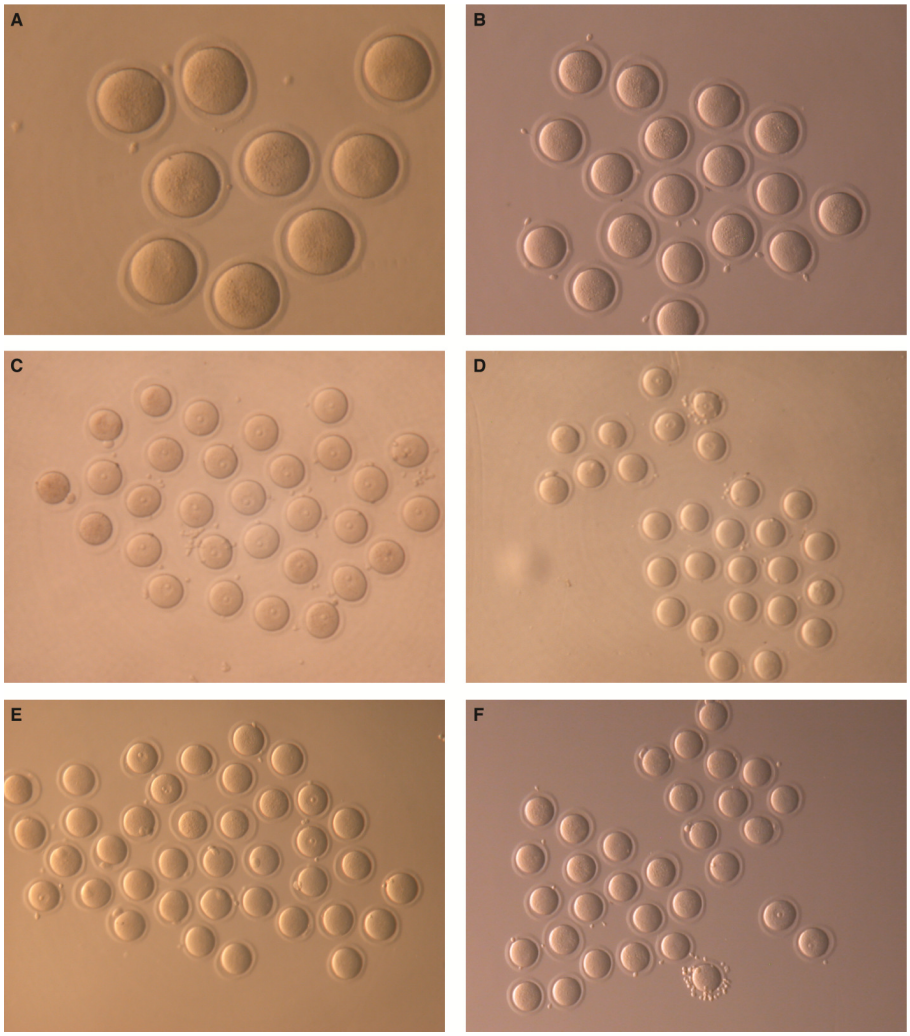

Figure 3.4. Ovulated oocytes with different meiotic developmental stage in early collected oocytes. Note the uniform MI oocyte ovulation in animals treated with 7.5 or 15mg CLZ at 4h pre-hCG (A and B, respectively). Note the ovulation of oocytes of mixed maturational stages of mainly GV and MI/MII oocytes from animals treated with 15 or 7.5mg CLZ at the same time as hCG (C and D, respectively). Note the ovulated oocytes of different maturational stages of mainly MII and MI oocytes and few GV oocytes form a female mouse treated with 15 or 7.5mg at 2h post-hCG (E and F, respectively).

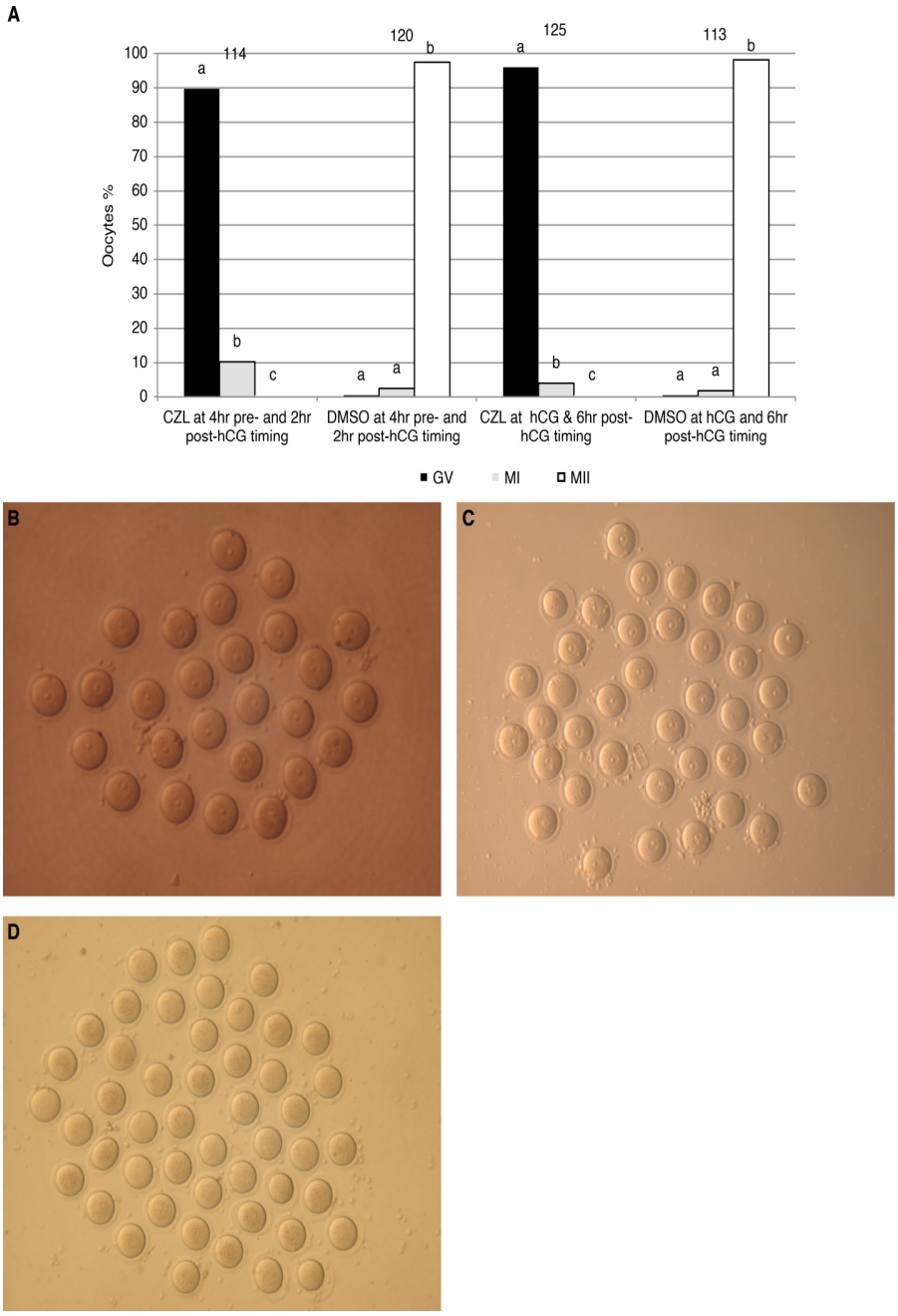

Figure 3.5. Multiple CLZ administrations in mice allow for GV oocyte ovulation upon early retrieval. Administration of 7.5mg CLZ in multiple doses at 4h pre-hCG and 2h post-hCG or same time as hCG and 6h post- hCG allowed for ovulation of GV oocytes. (A). Animals treated with 7.5mg CLZ at the same time as hCG and 6h post- hCG allowed a uniform GV oocyte ovulation upon retrieval (B). The same observation was noted in animals treated with 7.5 mg at 4h pre-hCG and 2h post-hCG (C). Delaying GV oocyte collection in animal treated with 7.5mg CLZ at 4h pre-hCG and 2h post-hCG resulted in MI oocyte collection (D). Data are presented as oocyte percent from the total number of oocytes. Values with no common superscript within each treatment time are significantly different ($p<0.05$) using the Mann-Whitney test.

Meiotic maturation initiation and continuation in the oviduct

In some experiments, oocyte collections from CLZ-treated animals were delayed until 14.5-15.5h post-hCG (late collection) to determine if ovulated immature oocytes at a specific meiotic maturation stage could initiate or finish meiotic maturation in this superovulated mouse model. The late collection of oocytes from animals treated with 7.5mg CLZ, 9h pre-hCG, showed a mean decrease in MI and an increase in MII oocytes when compared to those of early oocyte collections (1.8% *vs*. 38.5% and 98.1% *vs*. 61.3%, respectively, $P<0.0001$, for both, Fig. 3.6A). A similar observation was noted with the same doses but at 7h pre-hCG administration ($P<0.0001$, Fig. 3.6B), indicating the capability of ovulated immature oocytes at MI in animals treated with 7.5mg CLZ, 7 or 9h pre-hCG, to finish the process of meiotic maturation in the oviduct at this relatively short period of delayed collection. The scoring of ovulated GV and MI oocytes from late collection in animals treated with 7.5 or 15mg CLZ, 4h pre-hCG, revealed a significant increase in MI oocytes ($P<0.01$, for both doses) associated with a GV oocyte decrease ($P<0.01$ for 15mg treated mice) in comparison to those in early collection (Fig. 3.6C and D). These late collections displayed the ability of ovulated GV oocytes to start the process of GVBD in the oviduct and the inability of the ovulated MI oocytes to reach the MII stage within this time window of delayed collection. The initiation of GVBD and MI progression to the MII stage were observed together in late collections of oocytes from animals treated with 7.5mg CLZ at the same time as hCG (Fig.

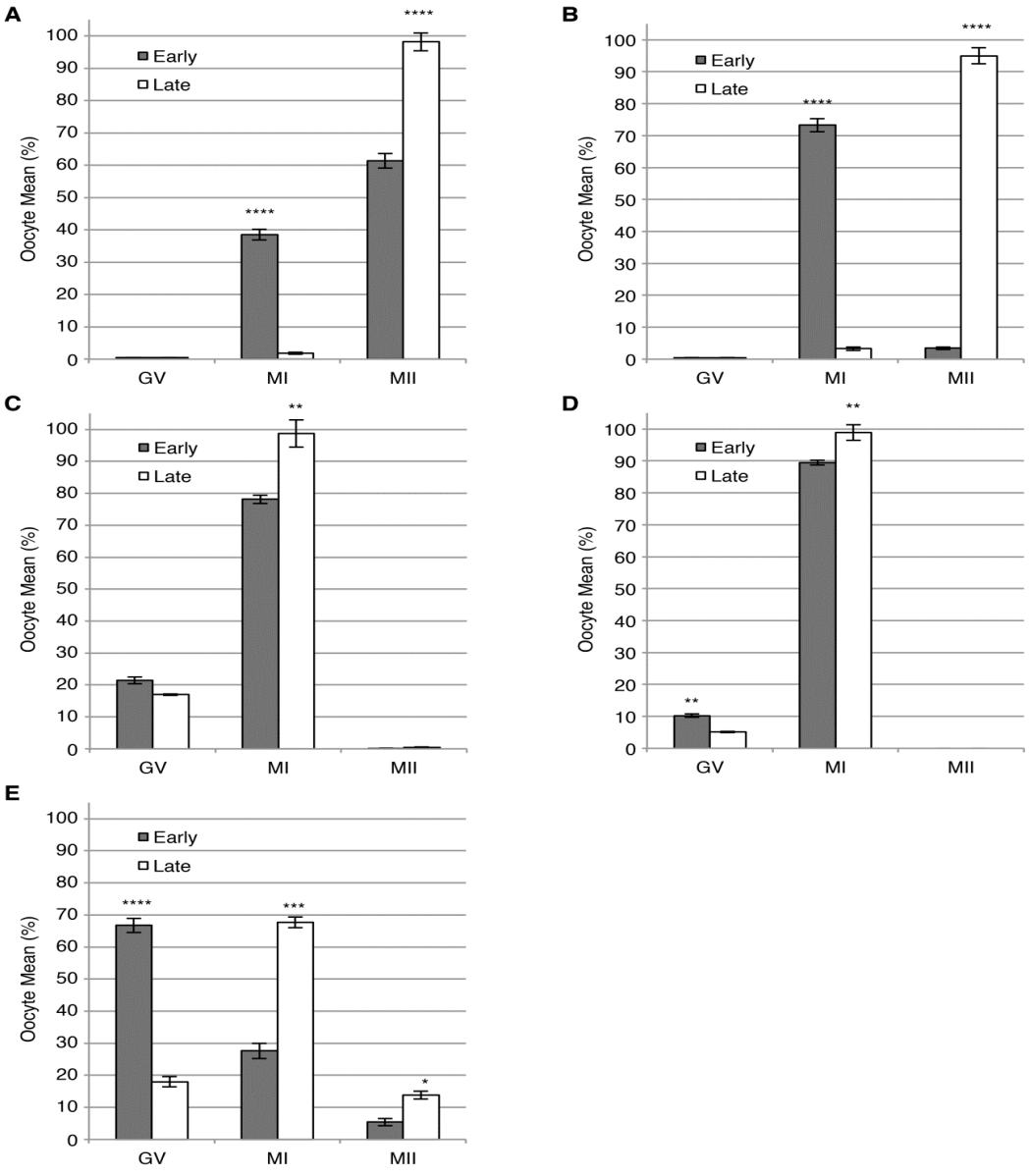

Figure 3.6. Late collection of ovulated oocytes from animals treated with CLZ allowed for some oocytes to resume and/or finish meiotic maturation. The late collection of oocytes from mice treated with 7.5mg CLZ dose, 9 h pre-hCG, led ovulated MI oocytes to finish maturation and reach the MII stage (A). The same observation was noted in 7.5mg CLZ treated animals, 7h pre-hCG (B). In animals treated with a single 15mg CLZ dose, 4 h pre-hCG, ovulated GV oocytes initiated meiosis and reached the MI stage in the oviduct at late collection (C). The same observation was noted in animals treated with single 7.5mg CLZ dose, 4h pre-hCG (D). Both ovulated GV and MI oocytes continued meiotic maturation to MI or MII, respectively in animals treated with 7.5mg CLZ dose at the same time as hCG (E). Data are presented as mean ± SEM percent. Differences were analyzed between the same oocyte maturation stage of early and late collections using Student T independent test. *<0.05, **<0.01, ***<0.001, and ****<0.0001.

3.6E). The ovulated GV oocytes underwent GVBD in late collected oocytes (66.75% *vs*. 18%, P<0.0001) which was accompanied by an MI oocyte increase (27.6% *vs*. 67.7%, P<0.001). Conversely, some of the ovulated MI oocytes were able to finish meiotic maturation at late collection and reached the MII stage (5.4% *vs*.13.8%, P<0.05). This phenomenon of concurrent events of GVBD and first polar body emission may be explained by the fact that both hCG and CLZ have similar onsets of action on follicles. As a result, some oocytes showed responses to hCG before attaining CLZ inhibitory plasma concentrations while others were blocked by CLZ. Figure 3.7 shows photographs of oocyte meiotic maturation in late collections from animals treated with different CLZ protocols.

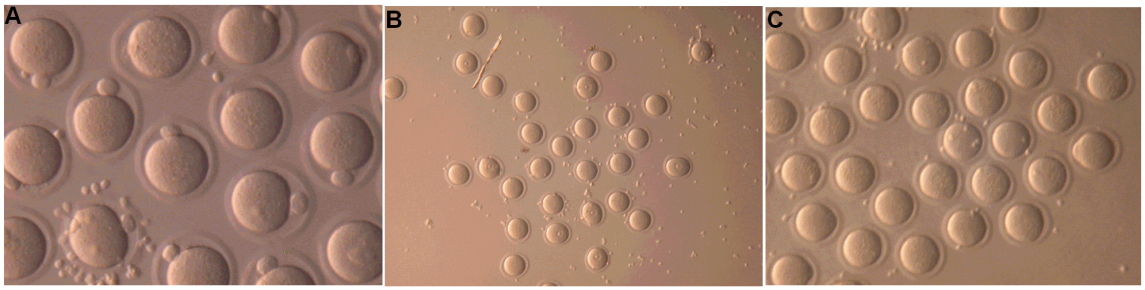

Figure 3.7. Delayed collection of ovulated oocytes in animals treated with CLZ allowed some of the immature oocytes to resume/finish meiotic maturation in the oviduct. Delayed oocyte collections from animals treated with 7.5mg CLZ, 9h pre-hCG, allowed the ovulated MI oocytes to reach MII stage (A). Animals treated with 15mg at 4h pre-hCG allowed a majority of GV oocytes to undergo GVBD when oocyte collection was delayed (B). Animals treated with 7.5mg CLZ at 4h pre-hCG allowed for the ovulated GV oocytes to undergo GVBD and for the ovulated MI oocytes to show signs of first polar body emission as indicated by arrows (C).

The ovulated GV oocytes (89.7%) in animals treated with 7.5mg CLZ, 4h pre-hCG and 2h post-hCG, were dramatically decreased at delayed collection to 32.2% ($P<0.0001$) accompanied with increased average numbers of collected MI oocytes (8% *vs*. 67.3%, $P<0.01$, Fig. 3.8A). The same case was recorded at late collection in animals treated with 7.5mg CLZ at the same time as hCG and 6h post-hCG but with a great variation (Fig. 3.8B). Four out of eight treated animals showed more than 85% GV oocytes at late collection while the other 4 showed mainly MI oocytes (standard deviations of 14.84 and 11.53 for GV and MI oocytes at late collection, respectively). Figure 3D show a photograph of uniform collection of MI oocytes at delayed collection.

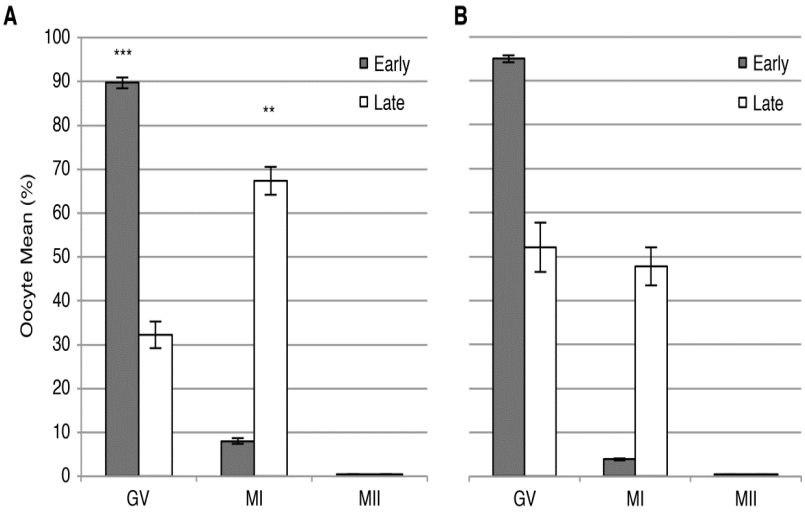

Figure 3.8: Delayed collection of ovulated oocytes in animals treated with multiple doses of CLZ allowed GV oocytes to resume meiotic maturation in the oviducts. Ovulated oocytes at early and late collections from animals treated with 7.5mg CLZ at 4h pre-hCG and 2h post-hCG (A) or at the same time as hCG and 6h post-hCG(B). Data are presented as mean ± SEM. Differences are analyzed between the same oocyte maturation stage of early and late collections.

GVBD monitoring in superovulated non-treated animals

In this experiment, we defined the time required for GVBD to occur in superovulated, non-CLZ-treated, the Swiss Webster mouse strain and found that 98% of the oocytes were at the GV stage surrounded by compact cumulus cells *vs*. 2% at the MI stage (P<0.0001) when oocytes were collected 3h post-hCG (Fig. 3.9A). This time-point showed the earliest time of oocyte GVBD to occur in this superovulated strain. Collection of oocytes at 6h post-hCG continued to show prevalence of GV oocytes over MI oocytes and MI oocytes over MII oocytes (69.3% *vs*. 28.1% *vs*. 1.98%, P=0.001 and P<0.0001, respectively). Most importantly, this time point-showed the first evidence of the earliest time that complete oocyte meiotic maturation can occur in superovulated mice. This phenomenon points to possible aging and viability reduction in oocytes that mature in a considerable time period (7-8h) before ovulation and also points to the importance of oocyte synchronization in superovulation protocols. Collection of oocytes at 6h post-hCG showed that more than 80% of the recovered oocytes were denuded or partially denuded (P<0.0001), while more than 90% of the EOs displayed cumulus expansions (P<0.0001). The recovery of denuded oocytes may be due to the loose and non-tenacious expanded cumulus cells that were lost during mechanical manipulation. Finally, aspirating oocytes after 9h post-hCG showed similar proportions of the three oocyte types with no significant differences. All those oocytes were surrounded with tenacious expanded

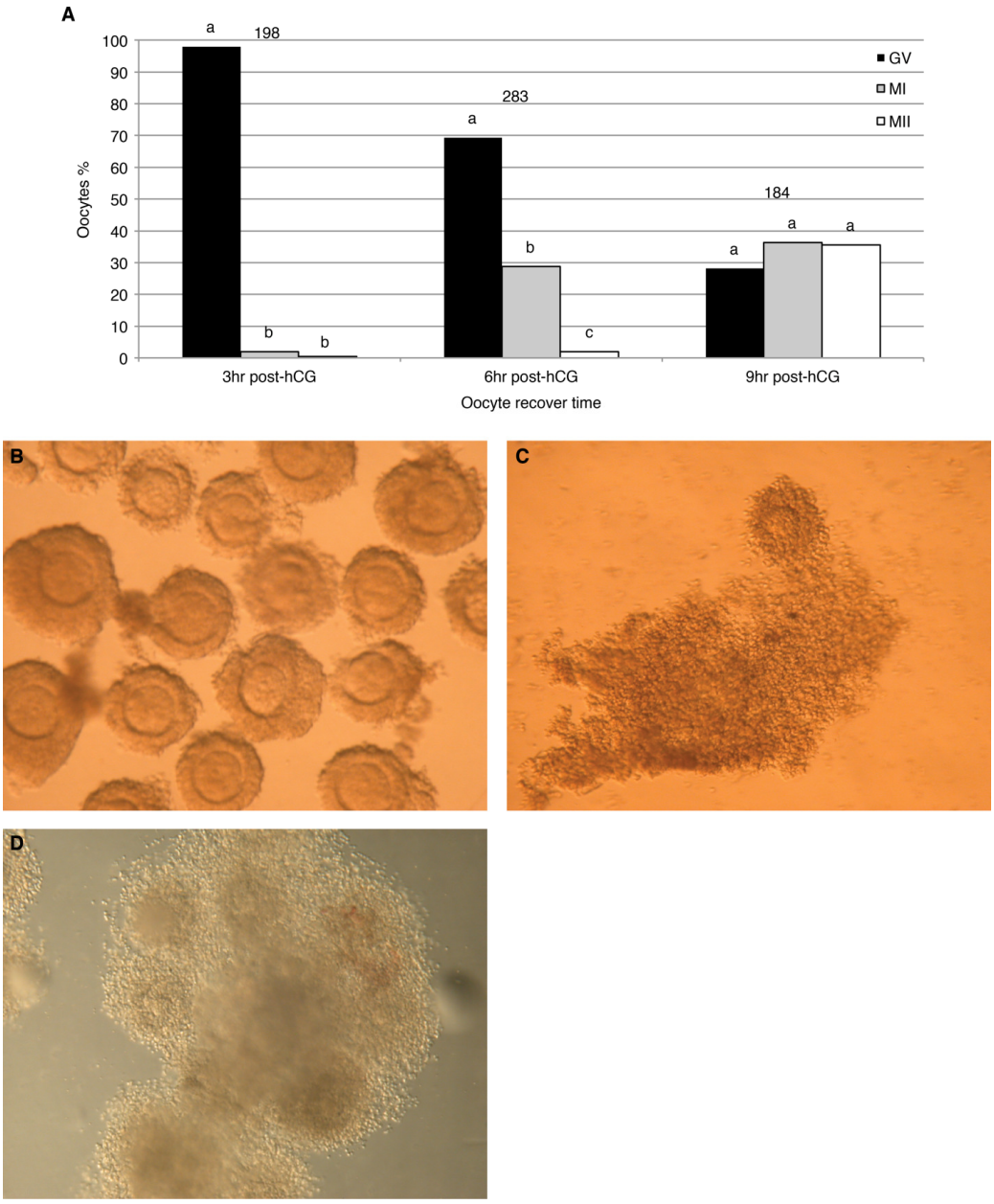

Figure 3.9. Monitoring oocyte meiotic maturation acquisition at different time points in superovulated mice. Animals were injected with ECG and after 47h with hCG. Oocytes were scored at 3, 6, or 9h post-hCG to define the time course of meiotic maturation acquisition after hCG (A). Ovarian oocytes surrounded by compact cumulus cells collected at 3h post-hCG (B). Ovarian oocytes collected at 9h post-hCG showing tenacious expanded cumulus cells (C). Note the oocyte complex aggregates upon ovulation (D). Data are presented as oocyte percent from the total number of collected oocytes. Number at the top of each collection time is the total number of oocytes collected. Values with no common superscript within each collection time are significantly different (p<0.05) using the Mann-Whitney test.

cumulus cells which might explain CEO complex aggregations and adhesions upon ovulation (Fig. 3.9B-D).

Cyclic cAMP level in ovulated immature oocytes

Cyclic AMP concentrations in ovulated GV oocytes were significantly lower than ovarian GV oocytes (P<0.0001). When pools of 40-65 oocytes per sample were evaluated, the content of cAMP from ovulated GV, MI, and MII oocytes were below the limit of the assay. Increasing the number of oocytes in the pools to 140-150 oocytes, resulted in the detection of cAMP in ovulated GV oocytes; however, by comparison, a markedly greater level of cAMP was detected from the smaller pool of 40-65 ovarian GV oocytes (Fig. 3.10).

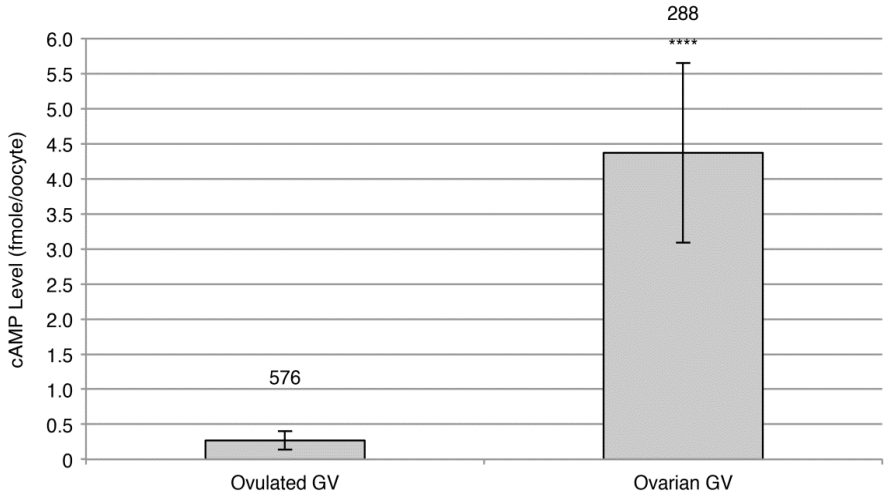

Figure 3.10. Cyclic AMP concentrations of ovulated and ovarian GV oocytes. Number at the top of each group is the total number of oocyte assayed. **** <0.0001 using the Student T test. Data are presented as mean±SD.

Discussion

We have previously shown for the first time that CLZ was able to block *in vitro* and *in vivo* oocyte meiotic maturation and pregnancy in mice (Taiyeb-Ridha *et al*. 2011a, b, and c) in concentrations and doses extrapolated from those in people who are taking CLZ. Here, we defined the timing of oocyte GVBD in superovulated Swiss Webster mice at 3 different time-points after hCG hormone injection and concluded that the process of GVBD was continuous from 3-9h post-hCG administration. We further controlled this breakdown via CLZ oral administration and allowed for GV, MI, MII, or mixed maturational stage oocytes to be ovulated based on the CLZ administration time, dose, and/or oocyte retrieval time. These protocols for retrieval of ovulated immature oocytes can be utilized as an additional research tool to the conventional method of ovarian collection of GV oocytes and subsequent *in vitro* maturation to obtain MI oocytes. The ovulated immature oocytes are characterized by high yield, easy retrieval, and uniform shape. On the other hand, these oocytes have lower levels of cAMP than the ovarian GV oocytes and are always surrounded by expanded cumulus cells and consequently lacked GJC.

In this study, we have also shown that the CLZ blocking property on oocyte meiotic maturation is reversible. Cilostazol delayed the process of GVBD, and once the inhibitory CLZ concentrations were assumed to decline, the oocytes were able to launch meiotic maturation in ovaries and/or the oviducts. The ability of CLZ to allow animals to ovulate MI oocytes when CLZ was

administered almost one day before ovulation time (such as the 9h pre-hCG) does not mean CLZ has a one day duration of action nor can block oocyte maturation at MI level because delayed collections allowed the ovulated MI oocytes to gain full meiotic maturation in the oviducts. It means that CLZ administration at 9 or 7h pre-hCG, is able to maintain a sufficient inhibitory concentration for the first few hours post-hCG, and this can briefly block the early effects of LH on oocyte GVBD. This short delay in the ovarian oocyte GVBD allowed the GV oocytes to have barely enough time in the ovary and/or reasonably enough time in the oviduct, to finish meiotic maturation. This can explain the ovulation of mixed MI and MII oocytes with the lack of GV oocytes collected early from animals treated with CLZ at 7 or 9h pre-hCG.

A similar hypothesis can be suggested for the ovulated GV and MI oocytes in animals treated once or twice with CLZ close to the time of hCG administration. At these times (such as the 4h pre-hCG and at the same time as hCG), we suggest that inhibitory concentrations of CLZ in blood may be maintained for longer periods after hCG as opposed to the earlier administrations of CLZ at 7 or 9h pre-hCG. These longer inhibitory CLZ periods, post-hCG, delayed the events that precede GVBD such that ovarian GV oocytes did not have enough time to fully resume meiotic maturation before ovulation; thus, leading to ovulation of GV and MI oocytes. Although these ovulated GV oocytes were able to undergo GVBD on delayed collections, the MI oocytes failed to emit the first polar body in the oviducts because this time window of

delayed collections was insufficient for this process to occur in those fresh MI oocytes (GVBD is thought to occur shortly before or after the ovulation). This demonstrates that the longer the arrest of ovarian oocytes at the GV stage, the more time required for the oviductal GV and MI oocytes to initiate the transition to the MI and MII stages, respectively.

Although the numbers of ovarian oocytes that underwent GVBD were low when oocytes were scored at 3 and 6h post-hCG injection, animals treated with CLZ 2 or 4h post-hCG injection ovulated MII oocytes. It is suggested that once the hCG effects on oocytes are triggered, the meiotic maturation is ultimately achieved. The hCG hormone has been shown to induce expression of epidermal growth factors (EGF) in preovulatory follicles (1h after being injected and reaching a peak after 3h). Those EGF family members are responsible for bringing the same morphological and functional events triggered by LH on follicles, such as cumulus cell expansion, GVBD (even when the spontaneous CEO meiotic resumption was arrested with PDE-I), and ovulation (Ashkenazi *et al*. 2005; Park *et al*. 2004). Follicles incubated with LH for 0.5-2h showed reduction of gap junction permeability; a phenomenon that precedes GVBD (Norris *et al*. 2008; Sela-Abramovich *et al*. 2008). Moreover, LH stimulation of cultured preovulatory follicles resulted in a decrease in the content of cGMP with nadir at 1.5h; cGMP is a nucleotide that maintains oocytes meiotically immature. The same trend of cGMP content reduction was observed in oocytes isolated 2h post-hCG in superovulated mice (Vaccari *et al*. 2009). These findings all point to

a rapid onset of action of LH that starts as early as 30min post-administration. Taken together, these results explain the inability of CLZ, when administered post-hCG, to prevent oocyte meiotic maturation. It is concluded that some meiotic maturational molecular events were triggered and initiated shortly after hCG administration, and PDE-Is are unable to forestall the ultimate meiotic maturation once the meiotic maturation is launched molecularly. It is also concluded that the early events of hCG on oocytes precede the morphological GVBD feature by 3-6h in this mouse strain.

It is important to direct attention to other factors, rather than the inhibitory CLZ concentration, that are influencing GVBD such as cumulus cell proliferation and expansion, GJC interruption, and cAMP concentrations of both somatic and germinal compartments. Those responses are direct consequences of hCG/LH hormonal effects on a selected cohort of follicles. Levels of GJC between the oocyte and somatic cells dramatically decreased upon mechanical isolation of the COC from the follicle. Although PDE-Is were shown to prolong GJC between the 2 compartments and to strongly block meiotic resumption (Dieci *et al*. 2011; Shu *et al*. 2008; Thomas *et al*. 2004), the LH and LH-like activity of hCG are believed to interrupt this communication prior to GVBD, to induce oocyte meiotic resumption, to decrease oocyte cAMP concentration, and to increase somatic cell cAMP production. This LH induced decrease in intraoocyte cAMP concentration, *in vivo* and *in vitro*, was found to be associated with interrupted follicular GJC and was not accompanied with elevated intraoocyte PDE3 activity,

suggesting follicular cAMP flow toward oocytes is important to arrest meiotic maturation (Hashimoto *et al*. 1988; Isobe *et al*. 1998; Norris *et al*. 2008; Sela-Abramovich *et al*. 2006; Sherizly *et al*. 1988; Vaccari *et al*. 2009). In addition to cAMP, cGMP has been shown to flow through the numerous gab junctions into oocytes to inhibit cAMP hydrolysis. The LH was observed to lower cGMP levels in the somatic cells, and consequently in the oocytes in addition to blocking GJC leading to the resumption of meiosis (Norris *et al*. 2009; Sherizly *et al*. 1988; Vaccari *et al*. 2009). Finally, although somatic cAMP when transmitted to the oocytes can prevent oocyte meiotic maturation, its elevation at the somatic compartment was confirmed to induce follicular maturation (Tsafriri *et al*. 1996). To put these observations into context with ours, the ovulated GV oocytes in animals treated with CLZ are believed to show the same GJC interruption and its consequences (such as low cAMP and probably cGMP concentrations) upon hormonal influence. Those conditions favor oocyte GVBD. The GVBD stimulatory factors can be challenged with steady state CLZ plasma inhibitory concentrations (Taiyeb-Ridha *et al*. 2011c). Nevertheless, it is still expected for ovulated GV oocytes retrieved from animals with sufficient circulatory CLZ concentrations to have barely enough intraoocyte cAMP to prevent GVBD but not as high as that in ovarian GV oocytes. It is concluded that a lower level of intraoocyte cAMP can maintain a state of GV in ovulated oocytes in comparison to the higher levels in ovarian GV oocytes. The ovarian GV oocytes have higher

concentrations of cAMP because of the active communication with the somatic compartment that is lacked in ovulated oocytes.

It is generally accepted that *in vitro* meiotic maturation of mouse oocytes derived from antral follicles takes approximately 18h, and the time duration required for *in vitro* GVBD is less than 4h. The spontaneous oocyte meiotic progression does not follow the pattern of *in vivo* oocyte meiotic maturation in superovulated females. Mouse strains differ in regard to time needed for oocytes to acquire meiotic maturation *in vivo* after the hCG/LH stimulus. Edwards and Gates (1959) reported that it required 4.5 to 8h for all oocytes to be in metaphase, while Calarco *et al*. (1972) found the GV oocyte type to be the predominant oocyte after 3h post-hCG injection using CF-I mice. Miao *et al*. (2004) showed a mean percent of 12.9% ±2.1 MII oocytes, recovered 8h post-hCG in Kun-ming mice. In contrast to these *in vivo* meiotic acquisition durations after the hCG injection, others reported GVBD *in vivo* of less than 4h post-hCG using different strains (Hsieh *et al*. 2007; Kalous *et al*. 2012). Although a uniform distribution of the 3 cell types was recorded only at 9h post-hCG in this research, almost all ovulated oocytes were mature with complete lack of GV oocytes in superovulated, non-treated, mice. Nevertheless, it is predicted that only MI oocytes at 9h post-hCG will proceed to the MII stage and to ovulate in addition to the already available MII oocytes at this time point. Adding those MI oocytes to the MII oocytes at 9h post-hCG was in agreement with our average oocyte yield at ovulation. It is also suggested that oocytes which fail to undergo GVBD

9h post-hCG will undergo atresia. In our CLZ protocols, we were able to retain the oocyte GVBD in a specified time frame based on the CLZ protocol used instead of this continuous GVBD in oocytes along the period post-hCG injection. This aids synchronization of oocyte meiotic development and prevention of early polar body emission with consequent aging and compromised oocyte quality and fertility (Taiyeb-Ridha and Kraemer 2011b).

From a contraceptive point of view, CLZ resulted in ovulation of a considerable number of MI oocytes in the superovulated mouse model when administered as far as 24h before ovulation. Moreover, CLZ blocked pregnancy in mated naturally cycling mice when administered daily (before and after mating), assuming ovulation of GV oocytes that failed to undergo meiotic maturation in the oviduct or gained meiotic maturation after long periods of arrest at the GV stage. Such oocytes would be aged, less viable, and incompetent (Taiyeb-Ridha *et al*. 2011c). Furthermore, the compound is relatively safe and is prescribed on a chronic basis in people with relatively advanced ages. The compound is indicated in people with IC disease, which is characterized by lower limb pain development upon walking due to selective femoral artery vasoconstriction. The unique secondary property of adenosine uptake inhibition, along with the primary PDE3-I mechanism, is believed to counterbalance the potential undesirable side effects of cAMP accumulation in platelets, vascular smooth muscle, and myocytes. This might explain the wide margin of safety of this compound over other PDE3-Is (Collins *et al*. 2005;

Kambayashi *et al*. 2003; Liu *et al*. 2001; Wang *et al*. 2001). These observations cumulatively suggest that CLZ is a potential and safe PDE3A-I contraceptive worthy of further studying and evaluation, even though the manufacturer of CLZ reported no observed impairment of fertility in rats (Otsuka America Pharmaceutical Inc. 2007; Sandoz Inc. 2004).

Overall, CLZ`s capability of blocking oocyte meiotic maturation *in vivo* was notable when the compound was administered at any time from 9h pre-hCG to 2h post-hCG. The compound is not different from other more potent PDE3-Is in regard to inhibition of oocyte meiotic maturation and is recommended for additional mammalian contraceptive studies. Also, we have presented evidence of oviductal-oocyte meiotic maturation which can be utilized as an *in vivo* culture system to synchronize oocyte meiotic maturation in superovulation. Finally, these tested CLZ regimens allowed for retrieving oocytes in different meiotic maturation stages with more convenience that can be considered as alternatives to the conventional methods of collection of meiotically immature oocytes from ovaries.

Summary and conclusion

- CLZ treatment results in ovulation of immature oocytes in superovulated mice.
- The evaluated CLZ doses in mice are similar to those indicated in humans for treatment of intermittent claudication.

- CLZ inhibits oocyte meiosis with a wide range of times of administration, which accounts for its contraceptive properties.
- Sustained high level and/or accumulation of cAMP in the arrested oocytes may explain the noted oocyte degeneration.
- Oocyte meiotic maturation in superovulated mice can be controlled when a PDE3-I is administered at anytime between 9h pre-hCG and 4h post-hCG; at least when the administered PDE3-I is CLZ.
- Collection of ovulated GV, MI, MII and/or oocytes with mixed maturational states is achievable via administration of CLZ in the superovulated mouse and this can substitute for conventional ovarian immature oocyte recovery.
- CLZ effect on meiosis is reversible, which can be utilized to synchronize oocyte meiotic and cytoplasmic maturation and progression *in vivo* and *in vitro*.
- Ovulated GV oocytes have lower cAMP concentrations than ovarian GV oocytes and as a result the ovulated GV oocytes can resume meiosis in a short period of time.

CHAPTER IV

CILOSTAZOL BLOCKS PREGNANCY IN NATURALLY CYCLING MICE

Overview

Incubation of immature oocytes with PDE3-Is was found to prevent oocytes from undergoing maturation. PDE3-Is were then evaluated *in vivo* and found to result in ovulation of immature oocytes. Concerns regarding inhibition of PDE3A that is expressed in heart and blood vessels discouraged further development of PDE3-Is as non-steroidal contraceptives. Cilostazol is a PDE3A-I that is approved for medical indications in humans and has an additional effect of adenosine uptake inhibition that is believed to counterbalance the undesirable outcomes resulting from PDE inhibition. The aim of this study was to determine the capability of CLZ to inhibit pregnancy using a naturally cycling mouse model. None of the mice treated with 7.5 or 15mg CLZ, BID, produced offspring whereas all of the control animals maintained pregnancy and delivered normal pups ($P<0.0001$). Remating of the females which had been previously treated with CLZ resulted in normal offspring and litter sizes that were similar to controls, indicating that the contraceptive effect is reversible at least after short term administration. It is concluded that CLZ is a potential non-steroidal contraceptive that merits further evaluation.

Introduction

In 1958, Sutherland and Rall reported the discovery and characterization of the first second messenger; cAMP from biological tissues. They also noticed a hydrolytic activity that was influencing the discovered nucleotide (Kresge *et al*. 2005; Rall and Sutherland 1958; Sutherland and Rall 1958). Not until four years later, Butcher and Sutherland (1962) purified and characterized the 1st PDE. During 70s and 80s, many other PDEs were discovered (Butcher and Sutherland 1962). PDEs were found to be expressed in almost all mammalian cells and organs. At the present time, PDEs are a large number of enzymes in at least 11 known distinct families and 21 different gene types (Bender and Beavo 2006). Among those are PDE3 and PDE4 that are expressed in oocytes and granulosa cells and are capable of hydrolyzing cAMP and cGMP, respectively, to produce the corresponding oocyte states of meiotic maturation (Tsafriri *et al*. 1996). Incubation of ovarian GV oocytes with PDE3-Is was found to efficiently arrest oocyte maturation at the GV stage. Although numerous experiments were conducted to examine this maturational inhibitory effect at the *in vitro* level, few experiments were conducted *in vivo* to study the possible utilization of PDE3-Is as potential therapeutic agents for birth control besides the current contraceptives. Wiersma *et al*. (1998) reported that PDE3-I suppresses oocyte meiotic maturation and consequent pregnancy without affecting ovulation and cyclicity in rodents. The evaluated PDE3-Is (cilostamide and Org 9935) decreased oocyte maturation without affecting the number of ovulated oocytes

(Wiersma *et al*. 1998). Other investigators (Jensen *et al*. 2005 and 2008; Jeffery *et al*. 2010) conducted several experiments in primates using Org 9935. While the compound was able to arrest oocyte meiotic maturation in superovulated monkeys and *in vitro*, it failed to block pregnancy unless an Org 9935 plasma concentration of 300nM was achieved (Jensen *et al*. 2002, 2005, and 2008; Jeffery *et al*. 2010). No further experiments were conducted to extend the potential contraceptive effects of PDE3-I *in vivo* despite the promising *in vitro* findings. The primary restriction for extrapolating the *in vitro* experimentations to *in vivo* trials is thought to be due to the wide expression of the PDE3A in many cell and tissue types including myocytes, platelets, and vascular smooth muscle (Bender and Beavo 2006). Inhibition of this enzyme in the oocytes is assumed to influence the same enzyme in other organs and consequently to cause undesirable cardiovascular adverse effects.

Cilostazol (Fig. 4.1) is one of the few PDE3A-I that has not been evaluated for such contraceptive purposes. The primary aim of this study was to evaluate CLZ, a quinolinone derivative that appears to be the safest of the PDE3-I compounds, as a potential non-steroidal contraceptive drug using a mouse model.

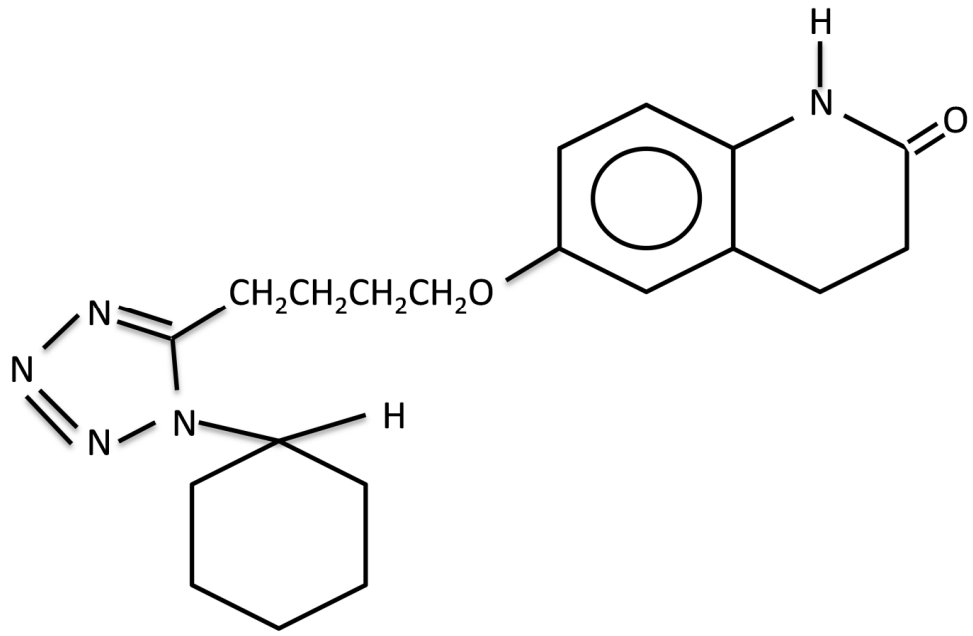

Figure 4.1. Cilostazol chemical structure. Cilostazol is a quinolinone derivative that has the chemical name 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3, 4-dihydro-2(1H)-quinolinone.

Materials and methods

Animals and reagents

Mature female and male Swiss Webster mice were purchased from Harlan Laboratories (Houston, TX) and housed in the Texas A&M University laboratory animal facility. Female and male mice of 5 and 6 months old, respectively, were kept in an air-conditioned room with controlled temperature and 14/10 h light/dark cycle. Food and water were provided *ad libitum*. All males used in this research were fertility proven whereas the females were virgin. The male fertility

test was conducted by mating each male to 2 females and recording litter size in the 2 mated females. Males were considered proven fertile when the females were pregnant and gave live births with normal litter sizes. All mice were identified by cage numbers and ear notches; 2 weeks before the start of the experiment. The average weights of mice treated with 7.5 mg CLZ, 15 mg CLZ, 0.15 ml DMSO, or 0.15 ml water were 25.95± 2.1 g, 25.3±3.2 g, 26.45±1.2 g, and 25.9±1.4 g, respectively. The animals from different groups were not significantly different in weight. All the experiments and procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee. Methylene blue and DMSO were purchased from Sigma (St. Louis, MO, USA) and CLZ was purchased from LKT Laboratories (St. Paul, MN, USA).

Monitoring estrus

Virgin female mice were checked for the stage and regularity of estrous cycles for 2 weeks by analyzing the vaginal cytology. Briefly, a small volume of normal saline was introduced into the vagina and aspirated back and forth for 2-3 times. The saline containing cells was placed on a slide, air dried, and stained with 1% methylene blue (of 70% methyl alcohol) for 5min. The slides were washed, dried, and analyzed under an Olympus IX71 inverted microscope. The metestrus phase of the estrous cycle was determined when leukocytes and nucleated and non-nucleated epithelial cells were detected in approximately equal proportions. Diestrus was determined when leukocytes were the predominant cell type,

whereas proestrus was identified by the presence of both nucleated and non-nucleated epithelial cells, with a small number of cornified cells. Estrus was verified when large numbers of cornified epithelial cells were observed. Forty females with 3-5 day estrous cycles were selected for this experiment and treatments began when females exhibited the first proestrus after the 2 week period of estrous cycle monitoring.

Cilostazol administration

Females were randomly assigned to four treatment groups: 7.5mg or 15mg CLZ, DMSO (vehicle control), or 0.15ml water (negative control). Cilostazol doses of 7.5 or 15mg were dissolved in 0.1 or 0.15ml DMSO, respectively. Cilostazol doses were prepared fresh just before administration. All treatments were administered orally by gastric gavage, twice a day, and each treatment group consisted of 10 females. Once the treatments were started, the vaginal smears were discontinued to exclude any possible interruption in mating. Females were placed with fertility proven non-treated males in a ratio of 1:1 three days after the initiation of treatments. Males were removed from the females when the seminal vaginal plug was detected. Treatments were continued until 1 day after detection of the vaginal plug. Each mated female was kept singly and was observed for 30 days for evidence of pregnancy and parturition. After the 30 days, each previously CLZ treated female was re-mated with the same male that was used before to examine the reversibility of the compound and to attribute any

contraceptive effect to the test compound and not to any other factor such as female infertility and/or unsuccessful mating. A new control group of 10 mice, non-treated previously, was used for the re-mating experiment to address possible side effects of CLZ on animal pregnancy and litter size. All resulted offspring were observed until weaning and later some were superovulated and others were naturally mated to assess normal reproductive function.

Statistics

The Fisher exact test was used to determine the statistical differences of pregnancies among treatments. The Student T test was used to determine the average litter size differences between DMSO and water treated groups.
The one way ANOVA was conducted to examine the differences among average litter sizes of re-mated animals and controls followed by the Games-Howell *post-hock* test. Differences were considered significant at $P<0.05$. The statistical software of SPSS 14.0 (SPSS Inc., Chicago, IL, USA) was used to conduct all the statistical tests.

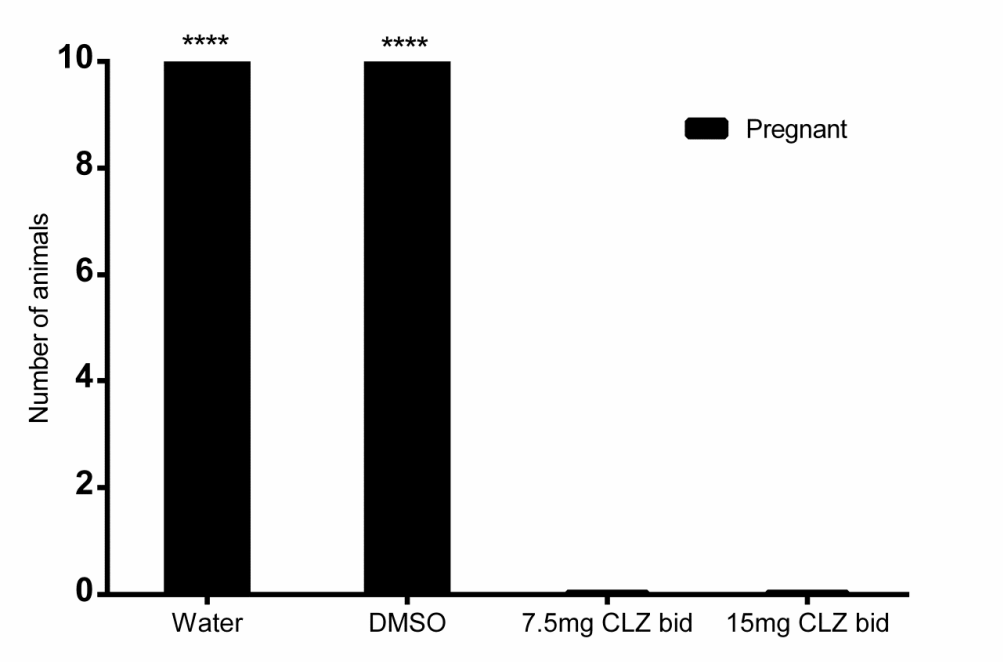

Figure 4.2. Effect of different treatments on pregnancy outcomes in naturally cycling mice. Note that none of the mice treated with CLZ produced pups in comparison to all of the control animals, which produced pups. Furthermore, DMSO did not affect pregnancy outcome (****<0.0001 using the Chi-square test).

Results

Figure 4.2 shows that none of the CLZ treated females conceived and produced offspring in comparison to all of the control females that conceived and produced offspring ($P<0.0001$). Furthermore, the average numbers of pups delivered by the dams that received water or DMSO were 8±1.4 *vs*. 6±1.6, respectively, $P>0.05$, indicating no direct detrimental effect of DMSO in this regard (Fig. 4.3). To assess CLZ reversibility, mice previously treated with CLZ

were re-mated 30 days after the first breeding. All of these females conceived and delivered normal pups and were not significantly different from controls (100% for each group). Figure 4.4 shows that there were no significant differences in the numbers of pups delivered among control dams and dams that were previously treated with 7.5 or 15mg CLZ, BID (7±1.18 *vs*. 6.41.35± *vs.* 7.6±0.91, respectively, p=0.11).

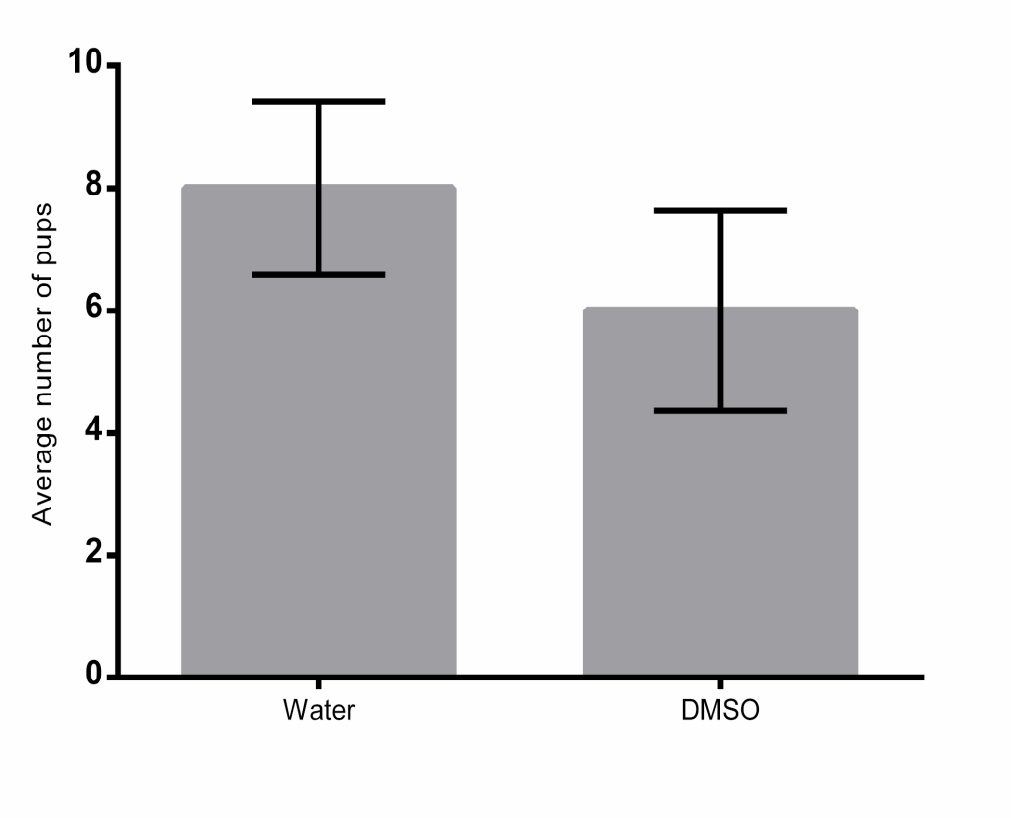

Figure 4.3. Effects of DMSO on litter size. Administration of the 0.15 ml DMSO vehicle control, bid, did not affect the litter size when compared to the water negative control (mean±STD, P>0.05 using the Student T test).

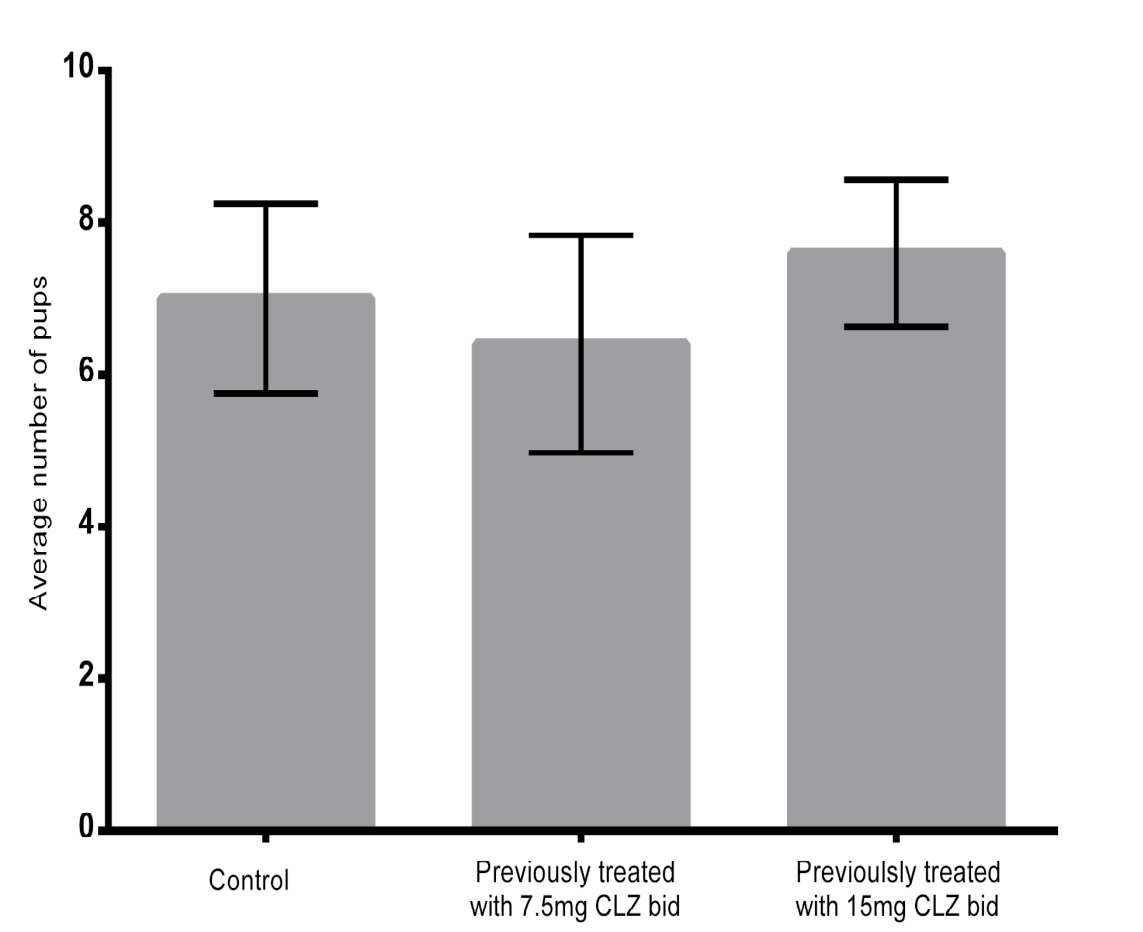

Figure 4.4. Reversibility effects of different CLZ treatments on litter size. The previously treated mice were re-mated with the same males used previously and allowed to complete the term. The number of pups per each female were counted and the average litter sizes were found similar among different groups (mean±STD, P>0.05 using the one way ANOVA).

Discussion

Previously, we found that CLZ was able to block oocytes from undergoing meiotic maturation *in vitro* and in superovulated mice (Chapter II and III). Here, we extended these observations to a more realistic state of contraception to

determine if the compound is able to block pregnancy. CLZ efficiently blocked pregnancy in naturally cycling female mice mated with fertile males that were not treated with CLZ. The notable contraceptive effect was reversible because all female mice previously treated with CZL became pregnant and had normal litter sizes. CLZ appears to be the safest of the PDE3-I compounds which, could be worthy of further evaluation as a non-steroidal contraceptive compound in other animal models. With the current steroidal contraceptive compounds, the benefits greatly outweigh the risks; nevertheless certain populations of people are vulnerable to undesirable health conditions and with family history of breast cancer and ischemic heart/cerebral diseases. Those conditions can be exacerbated with steroidal contraceptives, especially when used for long periods of time. This emphasizes the importance of further development of other alternative, non-steroidal, contraceptives suitable for those populations. The current steroidal contraceptive medications are known to precipitate side effects such as venous and arterial thromboembolism, hypertension, ischemic stroke. They are also associated with breast cancer (Beaber *et al*. 2012; Blanco-Molina *et al*. 2012; Hickson *et al*. 2011; Iatrakis *et al*. 2011; Tanis *et al*. 2001., White *et al*. 1994). It is suggested that CLZ shows fewer cardiovascular adverse effects than other PDE3-Is. CLZ, in comparison with other PDE3-I, has the ability to decrease adenosine uptake by muscle cells, endothelial cells, erythrocytes, and platelets. It also increases interstitial and circulatory adenosine concentrations that will moderately antagonize the positive intropic effect of CZL on the heart

(Liu *et al*. 2000 and 2001; Sun *et al*. 2002; Wang *et al*. 2001). Adenosine is thought to act on Adenosine-1 receptors (A1) on cardiac muscle leading to activation of Gi-protein and hence to inhibit adenylate cyclase (AC). Also, adenosine has been shown to attenuate protein kinase A-mediated increases in cardiac function and to decrease A-V automaticity and cardiotonicity of heart muscle produced by primary PDE3 inhibition via CZL. Adenosine acts on adenosine-2 receptors that activate Gs-protein to stimulate AC, and thus increases blood flow and inhibits platelet aggregation. Those effects are thought to be beneficial for women who use currently available contraceptives, which show cardiovascular and hematological concerns, especially in some populations (Liu *et al*. 2000 and 2001; Narayan *et al*. 2000; Nishi *et al*. 2000; Rang *et al*. 2007; Sun *et al*. 2002; Wang *et al*. 2001).

The compound, 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3, 4-dihydro-2(1H)-quinolinone, is an FDA approved therapeutic and is prescribed for intermittent claudication disease under the brand name of Pletal®. It is also available as a generic drug; cilostazol. It is interesting to mention that CLZ manufacturers stated in their CLZ drug leaflet/pamphlet that "*Cilostazol did not affect fertility or mating performance of female rats at doses as high as 1000 mg/kg/day"* (Otsuka America Pharmaceutical Inc. 2007). On the other hand, other PDE3-Is have been reported to block pregnancy of cycling rats and oocyte meiotic maturation *in vitro* and in superovulated mice and rats (Nogueira *et al*. 2003a; Tsafriri *et al*. 1996; Wiersma *et al*. 1998). In our study, we observed a

complete block of pregnancy (100%) in the two CLZ treated groups of mice in comparison to 100% pregnancy in the negative and vehicle control groups. It is possible either that the reproductive response to CLZ in mice is different from that in rats and/or the manufacturer`s method of administration of CLZ, masked some outcomes and observations. In the present research, CLZ was administered in divided doses, dissolved in DMSO, whereas the manufacturer administration method was a dietary administration method as specified under the section of carcinogenesis, mutagenesis, and impairment of fertility in the CLZ drug leaflet (Otsuka America Pharmaceutical Inc. 2007). In our study, the animal weight average for all of the 40 animals used was 25.9± 2.1g. This means that each of our CLZ treated mice would have had to be given approximately 25.9mg/day of CLZ based on the manufacturer`s highest tested dose of 1000 mg/kg/day in comparison to our tested doses of 7.5 mg, bid (579mg/kg/day) or 15mg BID (1158.3mg/kg/day). The manufacturer tested dose was higher than our tested dose of 7.5 mg twice a day, while our other tested dose of 15 mg twice a day, was slightly higher than the manufacturer tested dose. In both cases, CLZ blocked pregnancy in mice. The CLZ doses in this experiment were based on the *in vitro* inhibitory concentrations of CLZ on mouse oocyte maturation, which were made to be similar to plasma concentrations in people who are taking CLZ (Chapters II and III). Moreover, the possible effect of DMSO on fertility was excluded as DMSO vehicle was administered to mice and

did not affect animal fertility or mating performance. Further investigations are needed to clarify the contraceptive differences of CLZ between mice and rats.

Supplementation of the drug through diet may not be the best method to assess potential pharmacological and/or toxicological effects. Oral or parenteral active ingredient administration ensures specific drug amount intake in animals whereas food-dietary supplementation may not. Animal food appetite may vary among animals and within the same animal during the treatment period. Animal competitions and dominance/subordinance rank within a group can influence drug intake especially in multiple animal caging. An example of dominance/subordinance rank effects on housed animals was reported on housing male mice together. Males with high levels of testosterone dominated groups and suppressed testosterone concentrations in the subordinate ones (Machida *et al.* 1981). Moreover, the more densely housed groups showed markedly reduced food consumption and slightly decreased mean body weights (Chvédoff *et al.* 1980). This may have a considerable effect on animal appetites and access to food and influence therapeutic concentration in animals when drug intake is an animal responsibility. Moreover, food and fluid consumption is variable during the estrous cycle, which may add another interruption to drug level when drug administration is supplemented with diet (Michell 1979; Reddy and Kulkarni 1999). Rats showed elevated body weight and fat intake during diestrus when endogenous estrogens were reduced (Miceli and Fleming 1983). On the other hand, behavioral indices of anxiety were found to increase during

estrus day/hours in rats and mice (Mora *et al*. 1996; Palanza *et al*. 2001). Those factors collectively justify concerns about test compound administration in the dietary food at which many factors such as estrous stage and anxiety affect food intake. Also, animal dominance/subordinance rank may exaggerate the unstable drug delivery.

The New Drug Application (NDA) of CLZ reported to FDA indicated in the section of fertility study in rats that doses up to 1000 mg/kg/day, suspended in 0.5% carboxymethylcellulose and administered once daily via gavage, did not block pregnancy significantly. The pregnancy rates in all CLZ treated rats were as low as 87.5% in comparison to 95.8% control treated females (United State Food and Drug Administration/Center for Drug Evaluation and Research 1981). The later experiment was similar to our experiment with its delivery and route of administration but different with its duration of treatment, daily frequency of administration, and vehicle used. While the NDA reported administration of CLZ in rats for 14 days prior to pairing and continued through the pairing period until day 7 of gestation, we administered CLZ to mice for a an average number of 3-4 days in both CLZ treatment groups (United State Food and Drug Administration/Center for Drug Evaluation and Research 1981). Development of tolerance or changes in drug metabolism of CLZ in prolong administration have not been reported; however, sex differences were found to affect pharmacokinetics of CLZ in rats but not in mice nor in dogs. Oral administration of CLZ in female rats showed a greater increase in area under the curve and

bioavailability of CLZ than in males. These effects in females were accompanied with a reduction in a total body clearance of CLZ of one-sixth in comparison to that in males (Akiyama *et al*. 1985; Kamada *et al*. 2011). The reported experiment in the NDA also indicated a daily single oral administration of CLZ in rats in comparison to our daily divided administration of CLZ (BID). Although CLZ was documented to have two main active metabolites, high protein binding of 95 to 98%, and an elimination half life of 11-13hr in humans, the compound was reported to have a half life of 3.7 h in female rats upon10 mg/kg oral administration of 14C-cilostazol (Lacy *et al*. 2011). From our previous studies, CLZ was predicted to have an elimination half life in female mice relatively similar to that in humans as administration of CLZ at the time of hCG in superovulated mice showed mixed ovulation of GV and fresh metaphase I oocytes. Moreover, multiple administration of CLZ at 4h pre-hCG and 2h post-hCG or at the same time as hCG and 6h post-hCG showed ovulation of more than 90% of GV oocytes (chapter III). It is possible that single administration of CLZ in rats with relatively low half life of 3.7h may not be suitable to produce effects on fertility.

In addition, we used DMSO as a solvent and made CLZ solutions of 7.5 or 15mg CLZ dissolved in 0.1 or 0.15ml DMSO, respectively. The vehicle used in the NDA was 0.5% carboxymehtycellulose, which is known to be a suspender agent; hence, using this vehicle makes a suspension of CLZ. DMSO is known to easily cross biological membranes without altering their integrity. One of the

properties of DMSO is that it can facilitate penetration of its dissolved ingredient(s) through biological membranes and consequently increase bioavailability and effectiveness of the dissolved ingredient(s) (Jacob and Herschler 1986; Rammler and Zaffaroni 1967). It is possible that DMSO increased CLZ intestinal absorption and augmented its bioavailability and therapeutic effect higher than that of carboxymehtycellulose. Collectively, this information of using daily divided doses of CLZ, rather than single dose, oral (gavage) administration, rather than drug-dietary administration, and CLZ solution (especially when dissolved in DMSO), rather than suspension, might have shown a similar contraceptive effect in rats.

We are reporting here that CLZ is capable of blocking pregnancy in mice. Moreover, we have observed that CLZ was able to allow mice to ovulate significantly higher number of meiotically immature oocytes when CLZ was administered 9h pre-hCG to 2h post-hCG (Chapter III). Furthermore, we had observed previously that CLZ is capable of arresting oocyte meiotic maturation *in vitro* (Chapter II). More evaluations are recommended to test the potential contraceptive effect and safety of CLZ using other animal models.

Summary and conclusion

- CLZ blocks pregnancy in naturally cycling mice
- The evaluated doses are representative of those indicated in humans for therapeutic purposes.

- The doses evaluated by the manufacturer that did not impair fertility in rats were within our evaluated range of doses that impair fertility in mice.
- Differences in the effects of CLZ on fertility between mice and rats may be due to physiological differences, or to time, frequency, and/or method of administration.
- CLZ is a potential non-steroidal contraceptive that merits further evaluation

CHAPTER V

OVULATED IMMATURE OOCYTES HAVE ADVANCED DEVELOPMENTAL FEATURES IN COMPARISON TO OVARIAN IMMATURE OOCYTES IN MICE

Overview

Cilostazol was previously shown to result in ovulation of GV and/or MI oocytes in mice. Here, we compared cytoplasmic and meiotic maturation of ovulated GV and MI oocytes with corresponding ovarian GV and MI oocytes. Mice were superovulated and treated with CLZ to obtain ovulated GV and MI oocytes or were hyperstimulated to obtain ovarian GV oocytes. Some of the ovarian GV oocytes were incubated to obtain MI oocytes. Ovulated GV oocytes showed mainly chromatin configuration of surrounded nucleolus (SN) whereas the ovarian GV oocytes showed high rates of non-SN configuration ($P<0.0001$). Also, ovulated GV oocytes showed a higher incidence of peripheral cortical granules (CG) with intermittent domain in comparison to ovarian GV oocytes, which showed mainly a continuous peripheral CG distribution ($P<0.001$). Moreover, ovulated MI oocytes and MI oocytes obtained from *in vitro* maturation (IVM) of the ovulated GV oocytes had more normal spindles and chromosomes aligned at the metaphase plate than MI oocytes obtained from IVM of ovarian GV oocytes ($P<0.003$). Premature exocytosed CG were noted more in ovulated MI oocytes than other oocyte types ($P<0.05$). The GV oocytes were observed to have greater diameters than MI oocytes, and ovarian GV oocytes had greater

diameters than ovulated GV oocytes (P<0.007). Finally, ovulated GV oocytes showed higher rates of GVBD, MII, and IVF than ovarian GV oocytes (P<0.0001). These results indicate that ovulated immature oocytes have advanced developmental levels and consequently a higher fertilization rate. These ovulated immature oocytes may substitute for immature ovarian oocytes and become an additional research resource. More studies are needed to address their ability to produce offspring.

Introduction

Collection of GV oocytes from antral follicles in hyperstimulated mice is a widely established technique for retrieval of GV oocytes. Investigators use their experience to select antral follicles under a microscope before puncturing the follicle. This is sometimes followed by screening of oocytes based on morphology and diameter and usually mouse oocytes of less than 65-70μm are excluded, especially when the research is not designed to study outcomes from different oocyte sizes. Shortcomings with the current technique include oocyte yield because the numbers of antral follicles do not always represent the numbers of the ovulated oocytes, in addition to the possibility of collecting oocytes that are not from antral follicles. Moreover, ovarian GV oocytes show different types of chromatin configurations, CG and mitochondrial distributions, transcriptional activities, sizes, and others when collected after hyperstimulation (De La Fuente and Eppig 2001; Lee *et al*. 2006; Liu *et al*. 2005; Tokura *et al*.

1993; Wu *et al*. 2007; Zuccotti *et al*. 1995). Metaphase I oocytes are usually obtained via IVM of ovarian GV oocytes or directly from ovaries after administration of hCG or after the LH surge. The rate of MI oocytes obtained from IVM of ovarian GV oocytes depends on the type of culture medium, animal species, original size of GV oocyte/follicle used, cumulus cells state, and others (Choi *et al*. 2001; Curnow *et al*. 2010; Krisher 2004; Otoi *et al*. 2000; Schramm *et al*. 1993). Retrieval of MI oocytes directly from ovaries has the same limitations mentioned for the collection of ovarian GV oocytes, in addition to the need to determine the time course required for germinal vesicle breakdown (GVBD). While some investigators have reported a range of 4-9h post-ovulatory stimulus for GVBD to occur, others have reported times shorter than 3h for GVBD to occur depending on the mouse strain (Calarco *et al*. 1972; Edwards and Gates 1959; Hsieh *et al*. 2007; Kalous *et al*. 2006; Miao *et al*. 2004). In our experience, we have seen the time course of *in vivo* GVBD to vary within the same mouse strain at different times of the year even when mice are housed under controlled conditions.

Oocytes, upon isolation from follicles, have the ability to undergo meiotic maturation (Pincus and Enzmann 1934). Meiotic maturation is believed to occur before cytoplasmic maturation. The lack of time synchronization between those two maturational components is believed to cause deficiencies in oocyte development and competence. Oocytes collected in stimulated cycles are more readily fertilized after preincubation than are oocytes inseminated immediately

after collection. This is thought to be because of cytoplasmic maturation subsequent to the polar body extrusion (Sundstrom and Nilsson 1988). Moreover, temporal arrest of spontaneous meiotic maturation of GV oocytes *in vitro* using PDE3-Is is found to increase oocyte meiotic competence (Albuz *et al*. 2010; Nogueira *et al*. 2003a; Vanhoutte *et al*. 2007 and 2008).

Previously, we showed that oral administration of CLZ in superovulated mice resulted in ovulation of oocytes of different meiotic stages based on CLZ dose, frequency of administration, and times of administration and collection of ovulated oocytes. Administration of CLZ before hCG injection allowed superovulated mice to ovulate MI oocytes whereas administration of CLZ in divided doses around the time of hCG injection resulted in ovulation of GV oocytes. We also found that when collection of immature oocytes was delayed, the ovulated oocytes were able to resume meiotic maturation in the oviduct, indicating a temporal arrest of oocytes at the GV stage (Taiyeb-Ridha *et al*. 2011c). To define the quality of ovulated immature oocytes and to address the potential use of ovulated immature oocytes to substitute for ovarian immature oocytes, we studied meiotic and cytoplasmic maturation of the ovulated GV and MI oocytes and compared them with ovarian immature oocytes.

Materials and methods

Animals

Swiss Webster mice (8-10 weeks old) were purchased from Harlan Laboratories (Houston, TX, USA) and housed at the Texas A&M University Laboratory Animal Facility. Mice were under controlled temperature (23℃) and light/dark (14/10 h) cycle. Food and water were provided *ad libitum*. All experiments were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Reagents and chemicals

Bovine hyaluronidase, monoclonal anti- β -tubulin antibody produced in mice, and ethidium homodimer were purchased from Sigma (St. Louis, MO, USA) and CLZ was purchased from LKT Laboratories (St. Paul, MN, USA). Pregnant mare serum gonadotropin (Folligon®) and hCG (Chorulon®) were purchased from Intervet Inc (Summit, NJ, USA), and HTF (with or without HEPES) culture media for Murine IVF were obtained from Zenith Biotech (Guilford, CT, USA). Fluorescein lens culinaris agglutinin (LCA) and vectashield mounting medium with propidium iodide (PI) were purchased from Vector Laboratory (Burlingame, CA, USA). Alexa fluor 488 (ab`) 2 fragment of goat anti-mouse IgG (H+L), prolong gold DAPI mounting medium, and cell mask (red) were purchased from Molecular Probe (Grand Island, NY, USA). Lab-Tek II chamber slides were purchased from VWR International, LLC (Suwanee, GA, USA).

Experimental design

Experiments were designed to test the following hypotheses: 1) CLZ results in ovulation of GV oocytes with higher rates of surrounded or partially surrounded chromatin than ovarian GV oocytes. 2) CLZ results in ovulation of GV oocytes with higher rates of cytoplasmic maturation than ovarian GV oocytes as evaluated by CG distribution. 3) Ovulated GV oocytes have faster meiotic development and higher fertilization rates than ovarian GV oocytes upon IVM. 4) CLZ results in ovulation of more MI oocytes with normal spindles and chromosomes aligned at the metaphase plates than in those obtained from IVM of ovarian GV oocytes. 5) CLZ results in ovulation of more MI oocytes with advanced cytoplasmic maturation than those obtained from IVM of ovarian GV oocytes as evidenced by CG distribution. 6) Size of oocytes, GV nuclei, and nucleolus vary between ovulated and ovarian oocytes. Figure 5.1 shows the design of the experiments that were conducted to test these hypotheses.

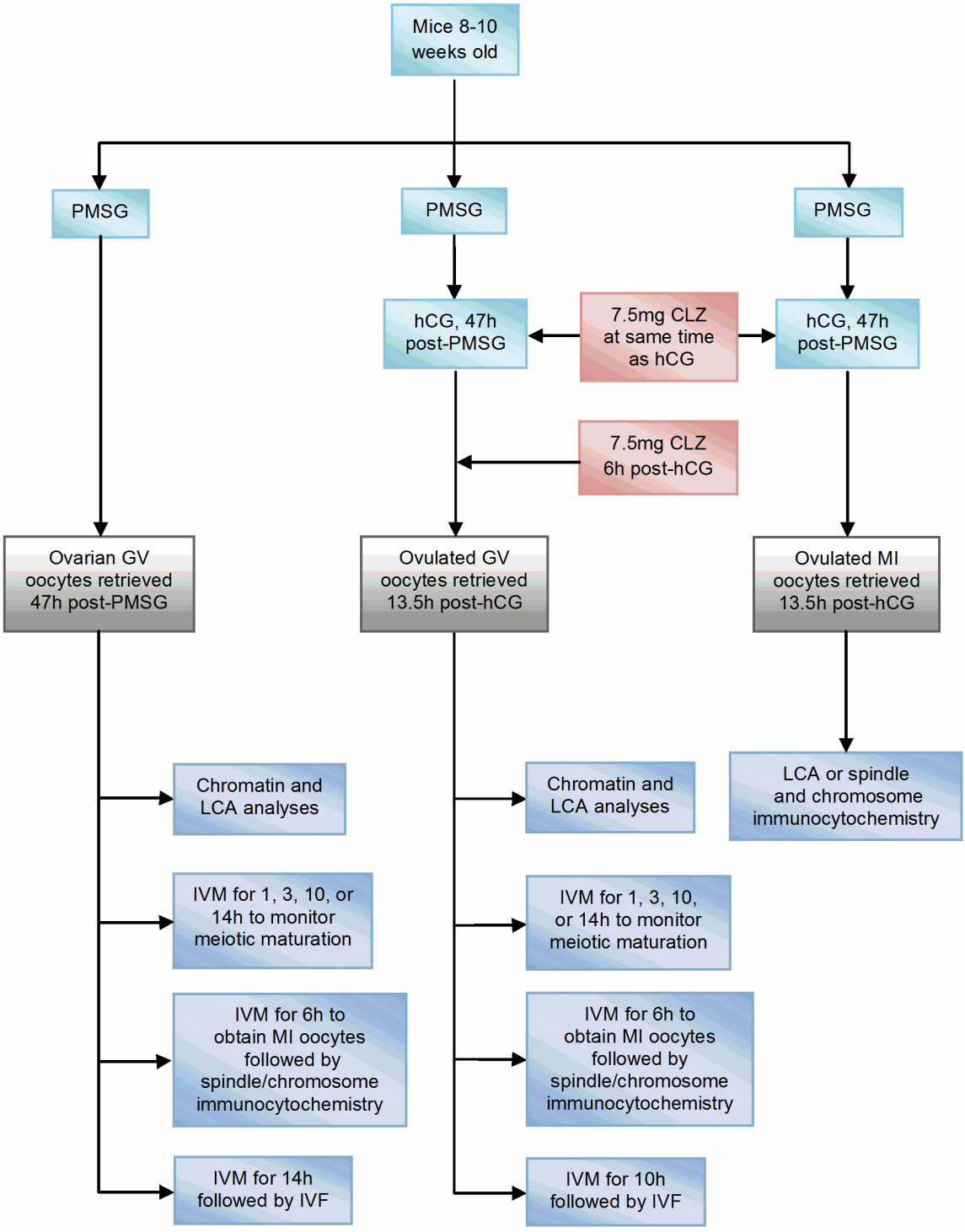

Figure 5.1. The experimental design for all the conducted experiments in chapter five.

Collection and processing of GV oocytes

Groups of mice were hyperstimulated with 10 IU PMSG, ip, and GV oocytes from antral follicles were isolated using a 30G needle. Other groups were superovulated with 10 IU hCG 47h post-PMSG, ip. All superovulated mice received 7.5mg CLZ dissolved in 0.1ml DMSO (gastric gavage) at the same time as hCG and 6h post-hCG to allow ovulation of GV oocytes (Taiyeb-Ridha *et al*. 2011c). The distended portion of the ampulla was punctured using a 26G needle to release oocytes. All GV oocytes (ovarian or ovulated) were collected in HTF medium with HEPES supplemented with 10% FBS and 4.2μM CLZ to block spontaneous maturation (Taiyeb-Ridha *et al*. 2011a). Some of the ovarian and ovulated cumulus enclosed GV oocytes were *in vitro* matured using HTF medium supplemented with 4.5% FBS for 1 or 3h, at 37℃ and 5% CO_2 in humidified air, to assess the time course and rate of GVBD.

Some of the ovulated GV oocytes were denuded using 0.006% hyaluronidase for less than 2min. Those oocytes in addition to the ovarian denuded GV oocytes were fixed in 4% paraformaldehyde for 20 minutes followed by permeabilization using blocking washing solution (BWS) three times for 10 minutes each. The BWS consisted of 1X phosphate buffered saline (PBS) without Ca^{2}+and Mg^{2}+, 0.2% non-fat dry milk, 0.01% triton X-100, 2% FBS, 0.1M glycine, 2% heat–inactivated normal goat serum, and 0.02% sodium azide and was filtered through a 0.2μm membrane. The oocytes were incubated with 2% LCA (diluted in PBS) for 30 minutes at room temperature followed by three

washes with BWS (each of 10min). The oocytes were then loaded on Lab-Tek II chamber slides with prolong gold antifade reagent with DAPI or vectashield mounting medium with PI. The slides were stored in the dark at 4℃ for at least three days to maximize signal: noise ratio. Oocytes were then analyzed for chromatin configuration and cortical granule distribution using a Carl Zeiss microimaging fluorescent inverted microscope (Stallion Digital Imaging Workstation; Thornwood, NY, USA) equipped with a C-Apochromat 63x water objective, a Photometrics CoolSnap HQ Camera, and slideBook software (Intelligent Imaging Innovations Inc.; Santa Monica, CA, USA). For FITC, an excitation filter of 470/20nm and emission filter of 505-530nm were used. Texas red data were collected with a 560/40 nm exciter and a 630/75nm filter. For cell mask, an excitation filter of 575/625nm and an emission filter of 660/710 were used to collect the data. DAPI data were collected with an excitation filter of 365nm and emission filter of 445/50nm.

Chromatin configurations were classified as follows: SN when chromatin formed a heterochromatin rim (intense fluorescent ring) condensed in close apposition with the nucleolus with or without few aggregates of condensed chromatin in the nucleoplasm area; partially surrounded nucleolus (PSN) when the nucleolus was partially surrounded by an opposed rim of condensed chromatin with few aggregates of condensed chromatin in the nucleoplasm area; and non-surrounded nucleolus (NSN) when chromatin was found non-condensed and homogenously diffused in the germinal vesicle (De La Fuente

2006; Debey *et al*. 1993; Nogueira *et al*. 2003a). The distributions of CG in the cytoplasm of GV oocytes were classified as previously mentioned with slight differences. GV oocytes were classified as diffused CG when the CG were observed throughout the cytoplasm, whereas they were classified as peripheral CG when the CGs were aligned at the cortical and subcortical regions. When the peripheral CG showed a clear region or domain of faded and non-continuous peripheral green florescence, it was classified as peripheral CG with intermittent domain (CGID). When the CG were not observed, observed on a small portion at the periphery, or at the zona pellucida, they were classified as exocytosed CG (Liu et al. 2003; Liu *et al*. 2005).

Collection and processing of MI oocytes

To obtain ovarian MI oocytes, cumulus enclosed GV oocytes collected from the ovaries of hyperstimulated mice were incubated in HTF medium supplemented with 4.5%FBS for 6h at 37℃ and 5% CO_2 in humidified air. Superovulated mice were treated with 7.5mg CLZ at the same time as hCG to result in ovulation of MI oocytes (Taiyeb-Ridha *et al*. 2011c). In mice that ovulated GV oocytes (as described in the previous section), ovulated cumulus enclosed GV oocytes were incubated as described for 3h to obtain MI oocytes. All resulted MI oocytes were fixed as described previously followed by blocking three times for 10 minutes each and incubation overnight with BWS at 4℃. On the next morning, oocytes were incubated for 1h at 37℃ with 1st antibody of 1:200 monoclonal anti- β -

tubulin produced in mice (diluted in PBS without Ca^{2}+and Mg^{2}+, 0.15 BSA and 0.15 Tween 20). Oocytes were washed five times in the BWS (10 minutes each wash) and then incubated for 1hr at 37C with the 2nd antibody of 1:200 of Alexa fluor 488 (ab`) 2 fragment of goat anti-mouse IgG (H+L). Oocytes were washed three times with BWS (20 minutes each wash). Other MI oocytes resulting from ovulation or IVM of ovarian GV oocytes were fixed and analyzed for CG distribution as described for GV oocytes. All of those MI oocytes that were prepared to study the spindle and chromosomal normality and alignment or CG distribution were loaded on Lab-Tek II chamber slides with prolong gold antifade reagent with DAPI or vectashield mounting medium with PI and stored for at least three days before analyses. Metaphase I oocytes were considered organized if they showed a normal spindle of barrel shape and chromosomes aligned at the spindle equator. Oocytes that showed asymmetric spindles, multipolar, or displaced chromosomes were considered disorganized (Ridha Albarzanchi *et al*. 2008; Roberts *et al*. 2005; Sanfins *et al*. 2003). The analysis of CG distribution in MI oocytes was made similar to that described in GV oocytes with one exception. Only when the peripheral CGs showed a clear region of free domain of CG were MI oocytes classified as oocytes with peripheral CG with free domain (CGFD).

Diameter measurements of oocytes

Vertical and horizontal diameters at the largest optical plane were obtained for GV and MI oocytes, GV nuclei, and nucleoli. Distances between the two poles of the spindles (spindle length) and of the equator of the spindles (spindle width) were obtained. Only those MI oocytes that displayed their spindles oriented along one optical plane (horizontal) were considered for length and width measurements. The use of Lab-Tek II slides provided a representative measurement for oocyte and organelle dimensions because the oocytes were not distorted or flattened by the coverslip and oocytes were allowed to maintain their spherical shape.

Collection and processing of MII oocytes

Ovulated or ovarian cumulus enclosed GV oocytes were incubated (as described above) for 10 or 14h to evaluate the time course and rate of MII oocyte maturation. For IVF experiments, ovarian cumulus enclosed GV oocytes were incubated for 14h whereas the ovulated cumulus enclosed GV oocytes were incubated for 10h followed by IVF. Sperm suspensions were prepared using spermatozoa collected from the tails of the epidiymides attached to ductus deferens from two mature males in HTF with HEPES culture medium supplemented with 4.5% FBS and incubated for 15 minutes at 37℃ in order for spermatozoa to capacitate. Spermatozoa were added to the dish containing oocytes from three females to make a final concentration of two million

spermatozoa/ml in the oocyte medium (Taiyeb-Ridha *et al*. 2011b). The oocytes were removed from the spermatozoa after 6h and washed three times with HTF supplemented with 4.5% FBS before incubating for 24h using the same medium. Oocytes were assessed for 2 cell embryo rate after 24h of incubation.

Statistical analyses

The Chi-Square test, or when appropriate, the Fisher`s Exact test, was used to analyze the differences in chromatin configuration, CG distribution, GVBD, MII, IVF, oocyte distribution along different size measurements, and spindle and chromosome assembly rates between/among different oocyte types and origins. The One-Way ANOVA (followed by the Tukey *post hoc* test) and the Independent-Sample T tests were used to analyze the differences among/between oocyte, GV nucleus, and nucleolus diameters of different oocyte types. The One-Way ANOVA analysis followed by the Games Howell *post hoc* test was also used to evaluate differences of spindle lengths and widths among MI oocytes obtained from different origins. The threshold for significant difference was set at $P<0.05$, and SPSS® 14.0 software (SPSS Inc., Chicago, IL, USA) was used to carry out the statistical analyses.

Results

Ovulated and ovarian GV oocyte evaluation

Chromatin configuration

The majority of ovulated GV oocytes showed SN chromatin configuration, with smaller proportions of oocytes demonstrating PSN configuration followed by NSN configuration (70.7% *vs*. 25.6% *vs*. 3.7%, respectively, P<0.0001, see Table 5.1). Conversely, the majority of ovarian GV oocytes showed NSN configuration as compared to PSN and NSN configurations (55% *vs*. 24.2% and 20.8%, respectively, P<0.0001). Ovulated GV oocytes showed a higher rate of SN configuration when compared to the ovarian GV oocytes (P<0.0001) whereas NSN configuration was noted more in ovarian GV oocytes than ovulated GV oocytes (P<0.0001). The rate of PSN configuration was not different between the two oocyte types. Table 5.1 shows chromatin configuration rates of ovulated and ovarian GV oocytes.

Table 5.1. Chromatin configurations of ovulated and ovarian GV oocytes.

Chromatin Configuration	Ovulated GV	Ovarian GV
SN (%)	70.7****	20.8
PSN (%)	25.6	24.2
NSN (%)	3.7	55****
Total number of oocytes	82	120

Sn: surrounded nucleolus, PSN: partial surrounded nucleolus, NSN: non-surrounded nucleolus. Values with asterisks are significantly different along each row where ****<0.0001.

Cortical granule distribution

Ovulated GV oocytes showed mainly peripheral CG with or without intermittent domain more than other distributions (P<0.003, see Table 5.2). These two distributions were followed by diffused CG, which was in turn followed by exocytosed CG (15.85% *vs*. 1.21%, respectively, P<0.0001). In ovarian GV oocytes, peripheral CG was the dominant distribution (P<0.0001), and diffused CG was the next most common, with other distributions being less common (P<0.0001). Comparing the CG distribution between the two types of GV oocytes revealed that the ovarian GV oocytes have a higher incidence of peripheral CG distribution whereas the ovulated GV oocytes have a higher incidence of peripheral CGID (P<0.001). Table 5.2 shows the CG distribution of ovulated and ovarian GV oocyte types.

Table 5.2. Cortical granule distributions of ovulated and ovarian GV oocytes.

	Ovulated GV	Ovarian GV
Diffused CG (%)	15.85	22.68
Peripheral CG (%)	47.56	71.42***
Peripheral CGID (%)	35.36****	4.2
Exocytosed CG (%)	1.21	1.68
Total number of oocytes	82	119

CG: cortical granules, CGID: cortical granules with intermittent domain, ECG: exocytosed cortical granules. Values with asterisks are significantly different along each row where ***<0.001, ****<0.0001.

Coincidence of chromatin configuration and CG distribution

Analysis of CG distributions (as an indicator of cytoplasmic maturation) among the different chromatin configurations (as an indicator of meiotic development) was conducted. It was observed that the ovarian GV oocytes whether of SN, PSN, or NSN chromatin configuration had peripheral CG more than other distributions ($P<0.03$, Fig. 5.2A). It is concluded that the CG distribution in ovarian GV oocytes is independent of chromatin configuration because all the different configurations showed mainly CG distributed at the periphery of the oocytes.

Ovulated GV oocytes of SN configuration displayed mainly peripheral CG with or without intermittent domain compared to other distributions ($P<0.0001$, Fig. 5.2B), whereas ovulated GV oocytes of PSN configuration did not show a dominant distribution of CG. Since only three ovulated GV oocytes displayed NSN configuration (one for each of diffused CG, peripheral CG, or peripheral CGID) out of 81 ovulated GV oocytes, they were excluded from this comparison. These results indicate that the ovulated GV oocytes of SN configuration are relevant to both peripheral CG with or without intermittent domain. Moreover, the advanced stage of CGID, which is a sign of the beginning of formation of the free domain that usually occurs in MI oocytes, indicates that GV oocytes of SN configuration begin to initiate meiotic maturation. Increasing the number of ovulated GV oocytes with PSN configuration (more than 21 oocytes) might provide an accurate interpretation for the lack of a similar trend with ovulated GV

oocytes. Figure 5.3 shows photos of different chromatin configurations and CG distributions of ovarian and ovulated oocytes.

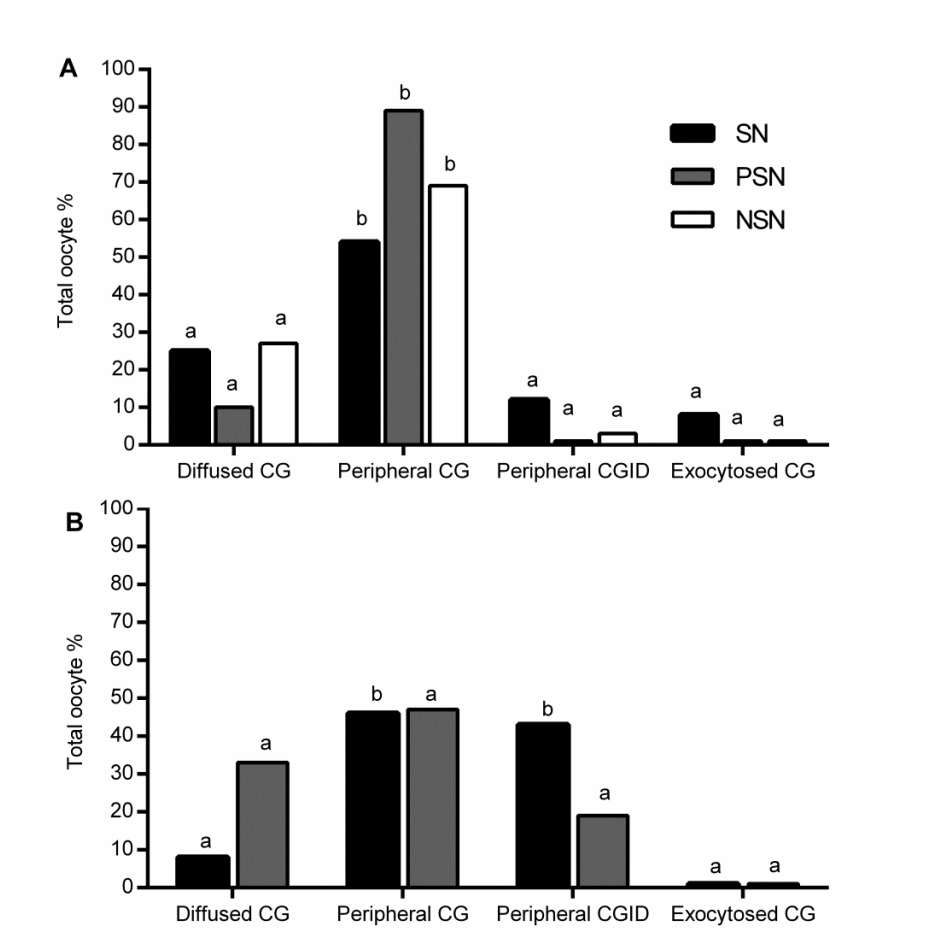

Figure 5.2. Ovarian and ovulated GV oocytes of different configurations of chromatin within different distributions of cortical granules (CG). A: ovarian GV oocytes. The numbers of oocytes that were considered for both chromatin and CG configurations were as follows: SN= 24, PSN= 29, NSN= 66. Different superscripts of the same chromatin configuration along different distributions of CG are significantly different $P<0.03$. B: ovulated GV oocytes. The numbers of oocytes that were considered for both chromatin and CG configurations were as follows: SN= 60, PSN= 21. Only 3 ovulated GV oocytes were with NSN configuration and were not considered in this graph (1 GV oocyte for each of diffused CG throughout cytoplasm, peripheral, or peripheral CG with intermittent domain). $P<0.0001$.

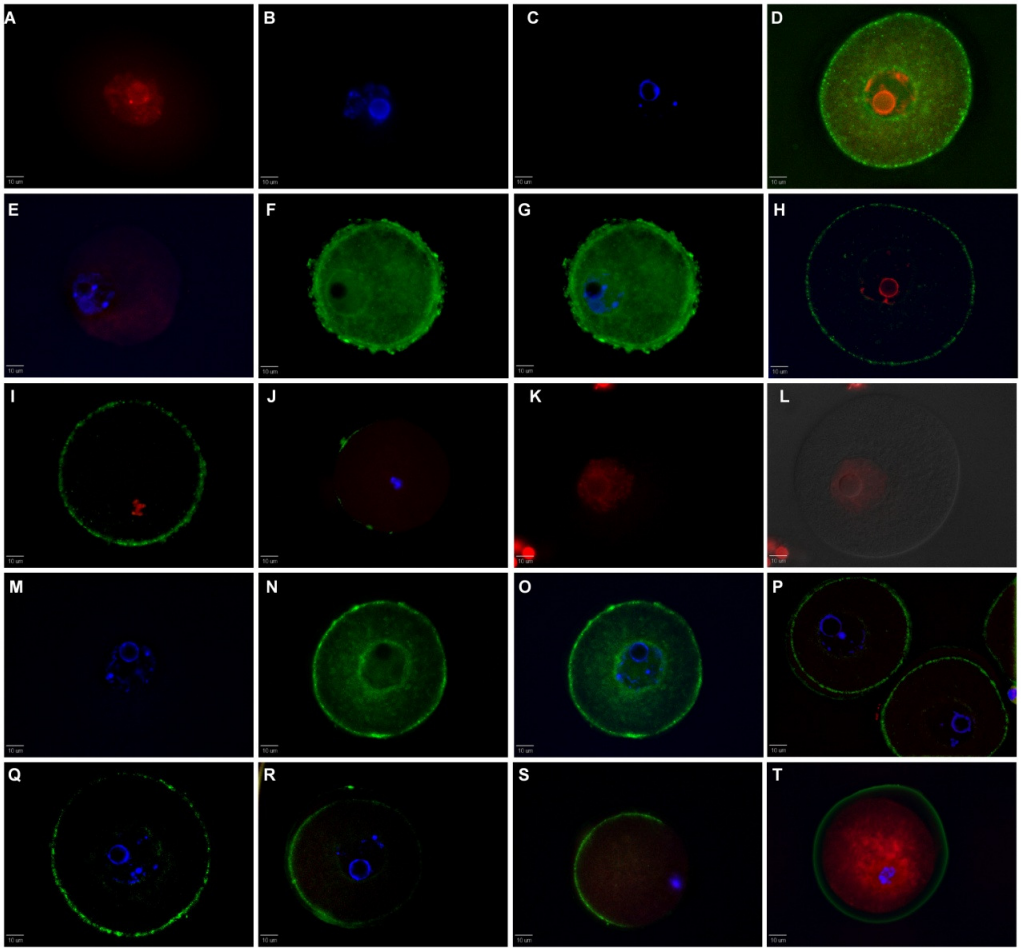

Figure 5.3. Ovulated and ovarian immature oocytes with different chromatin and CG levels of maturation. A-J are pictures of immature ovarian oocytes. A: is a GV oocyte with NSN configuration detected with PI. Note the non-condensed and homogenously diffused chromatin in the germinal vesicle. B: is a GV oocyte with PSN configuration detected with DAPI. Note that not all of the chromatin has condescended. C: is a GV oocyte with SN configuration. Note the formation of the rim with 2 condescended chromatin foci. D: is a GV oocyte with PSN and diffused CG. Note the distribution of CG throughout the cytoplasm. E-F: is a GV oocyte with NSN configuration (E) and diffused CG (F). G: is the merged picture of E and F. H: is a GV oocyte with SN configuration and peripheral CG. Note the distribution of CG at the periphery. I-J: are MI oocytes obtained form IVM of ovarian GV oocytes. Note the peripheral CG distribution (I) and exocytosed CG (J). K-T: are pictures of ovulated immature oocytes. K: is a GV oocyte with NSN configuration. L: is the merged picture of K. M-N: is a GV oocyte with PSN configuration (M) and diffused CG (N). O: is the merged picture for M and N. P: is a picture for two GV oocytes of SN configurations and peripheral CG. Q-R: are GV oocytes of SN configurations and peripheral CGID. S: is an ovulated MI oocyte with peripheral CGFD. T: is an ovulated MI oocyte with exocytosed CG. Note the distribution of CG at the zona pellucida while the oocyte is stained with the cell mask (red).

Meiotic maturation

Scoring of ovulated and ovarian cumulus enclosed GV oocytes after 1h of IVM showed a higher incidence of GVBD in ovulated oocytes than ovarian oocytes (90.9% *vs*. 10%, respectively, P<0.0001, Table 5.3). Actually, we observed GVBD in ovulated GV oocytes happening within a few minutes when CLZ was not added to the initial collection medium (data not included). Scoring of cumulus enclosed oocytes incubated for 3h showed 99% GVBD in ovulated GV oocytes and 86.3% GVBD in ovarian GV oocytes (P<0.0001). We further extended the IVM for 10h and scored other groups of cumulus enclosed oocytes and found that 97.27% of the ovulated GV oocytes had emitted the 1st polar body whereas 61.6% of the ovarian GV oocytes had emitted the 1st polar body. Extending the incubation to 14h in other groups of GV oocytes continued to show low rates of MII oocytes resulting from ovarian GV oocytes in comparison to those observed in ovulated GV oocytes (P<0.0001). Incubation beyond 14h did not improve MII rate for ovarian cumulus enclosed GV oocytes (data not shown). Therefore, ovarian or ovulated cumulus enclosed GV oocytes were incubated for 14 or 10h, respectively, followed by IVF. The ovulated GV oocytes showed a higher rate of 2 cell embryos than ovarian GV oocytes (84.81% *vs*. 68.31%, respectively, P<0.0001, Table 5.3).

Table 5.3. Meiotic maturation and *in vitro* fertilization rates of ovulated GV and ovarian GV oocytes.

	Ovulated GV, n (%)	Ovarian GV, n (%)
GVBD-IVM/1h	120/132 (90.9)****	12/120 (10)
GVBD-IVM/3h	92/93 (99)***	88/102(86.3)
MII- IVM/10h	143/147 (97.27)****	74/120 (61.6)
MII- IVM/14h	99/100 (99)****	94/112 (84)
IVF	134/158 (84.81)****	138/202 (68.31)

GVBD: germinal vesicle breakdown, IVM: *in vitro* maturation, MII: metaphase II, IVF: *in vitro* fertilization. Values with asterisks are significantly different along each row where ***<0.001 and ****<0.0001.

Ovulated and in vitro *matured MI oocytes*

Spindle and chromosomal organization

Ovulated MI or MI oocytes resulting from IVM of ovulated GV oocytes showed higher percentages of normally organized spindles with chromosomes aligned on the metaphase plate than those of MI oocytes obtained from IVM of ovarian GV oocytes (88.4% and 93.9% *vs*. 70%, respectively, P<0.003, Fig. 5.4A). We evaluated the length and width of spindles only in oocytes that were classified normal. We found the spindle width of MI oocytes obtained from IVM of ovarian GV oocytes longer than those in MI oocytes obtained from IVM of ovulated GV oocytes and ovulated MI oocytes (12.7±3.8µm *vs*. 11.2±2.2µm and 9.9±3µm, mean±SD, respectively, P=0.002, Fig. 5.4B). The spindle lengths were not significantly different (P=0.33). Figure 5 shows MI oocytes with different spindle and chromosomal organizations.

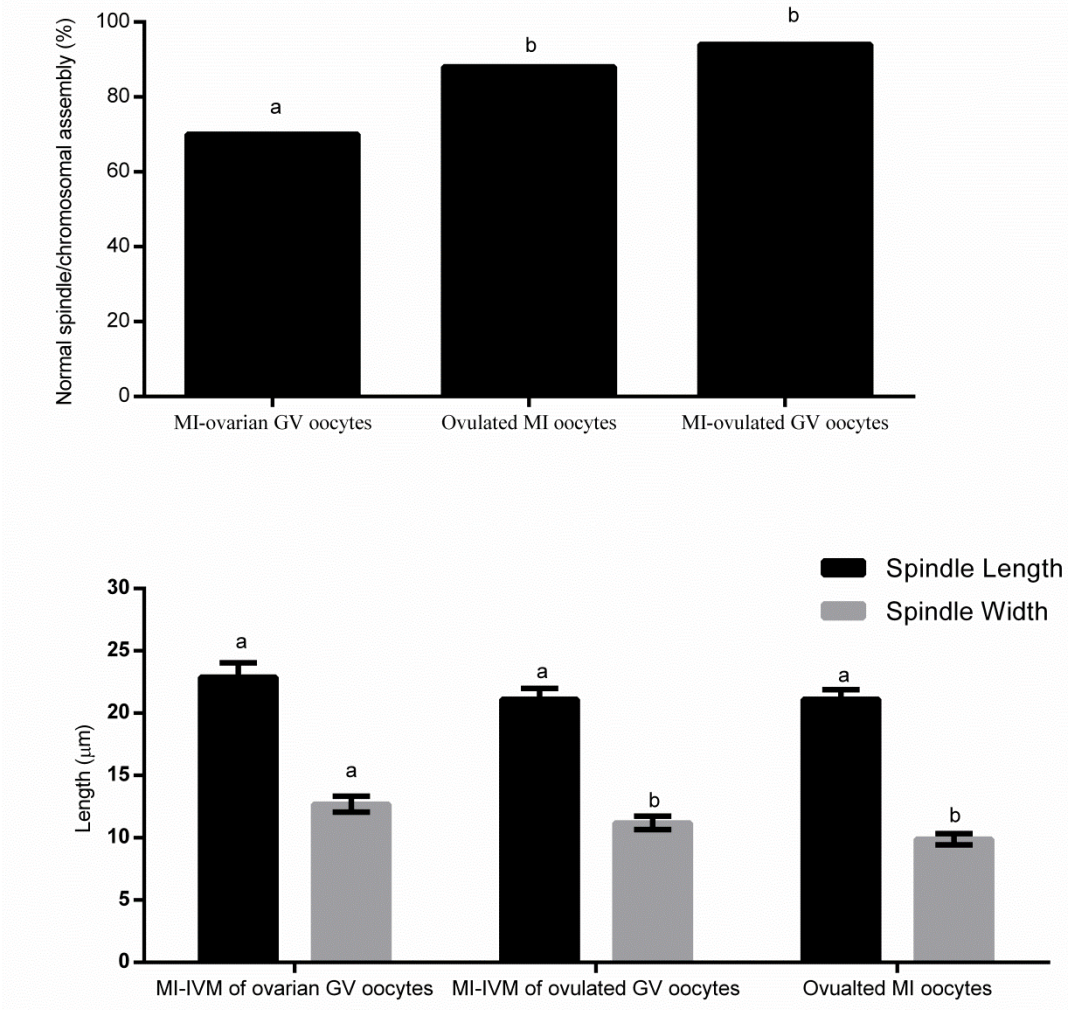

Figure 5.4. Spindle and chromosomal organizations and dimensions in MI oocytes collected from different protocols. A: Ovulated MI oocytes (95 oocytes) and MI oocytes (33 oocytes) obtained from IVM of ovulated GV oocytes showed higher rates of organized spindles with aligned chromosomes at the spindle metaphase plate when compared to MI oocytes (90 oocytes) obtained from IVM of ovarian GV oocytes. Different superscripts are significantly different ($P<0.003$, Tukey *post hoc* test). B: Spindle length did not vary significantly among MI oocytes from different origins but MI oocytes obtained from IVM of ovarian GV oocytes showed wider spindles at the equator than other MI oocyte types (mean±SEM). Different superscripts of spindle length or width along different MI oocyte types are significantly different ($P=0.003$, ANOVA-Games Howell *post hoc* test).

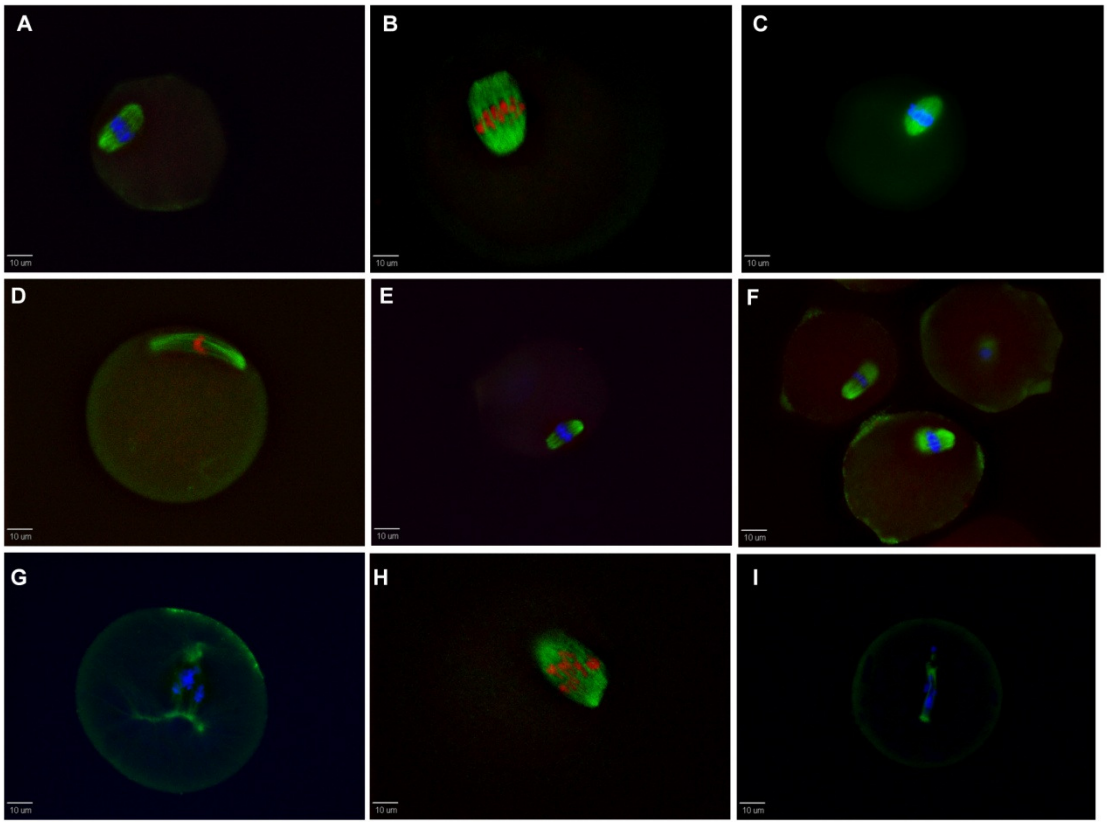

Figure 5.5. Various spindle and chromosomal organizations of MI oocytes obtained from different methods. A, D, and G are MI oocytes collected from IVM of ovarian GV oocytes. A displays normal spindle and chromosomal organization whereas D and G show abnormal spindle morphology. B, E, and H are MI ovulated oocytes. B and E show normal spindle and chromosomal organization whereas H has chromosomes that are not aligned at the spindle equator. C, F, and I display MI oocytes collected from IVM of ovulated GV oocytes. C and F display normal spindle and chromosomal organization whereas I shows an MI oocyte with abnormal spindle and chromosomes displaced from the equator.

Cortical granule distribution

MI oocytes obtained from IVM of ovarian GV oocytes showed a high rate of peripheral CGFD ($P<0.003$) followed by peripheral CG ($P<0.005$) when compared to other distributions (Table 5.4). Ovulated MI oocytes showed a similar trend but the rate of oocytes with exocytosed CG was significantly higher

than for oocytes with diffused CG (11.6% *vs.* 2.1%, P=0.01). The distribution of CG between the two MI oocyte types were similar except for the high incidence of exocytosed CG in ovulated MI oocytes in comparison to MI oocytes obtained from IVM of ovarian GV oocytes (11.6% *vs*. 3.9%, respectively, P<0.05, Table 5.4).

Table 5.4. Cortical granule distributions of ovulated MI oocytes and MI oocytes obtained from IVM of ovarian GV oocytes.

	MI oocytes from IVM of ovarian GV oocytes	Ovulated MI oocytes
Diffused CG (%)	7.8	2.1
peripheral CG (%)	29.4	26
Peripheral CGFD (%)	58.8	57.3
ECG (%)	3.9	11.6*
Total number of oocytes	51	96

CG: cortical granules, CGFD: cortical granules with free domain, ECG: exocytosed cortical granules. Values with asterisks are significantly different along each row where *<0.05

Diameter measurements of oocytes

A majority of the ovulated GV oocytes have diameters within 70.1-80µm followed by diameter ranges of 60.1-70µm and 80.1-90µm (P<0.002) followed by other sizes (P<0.0001, Fig. 5.6A). For ovarian GV oocytes retrieved from antral follicles, more oocytes with a diameter range of 90.1-100µm were observed than other diameter ranges (P<0.01) but was similar to oocytes

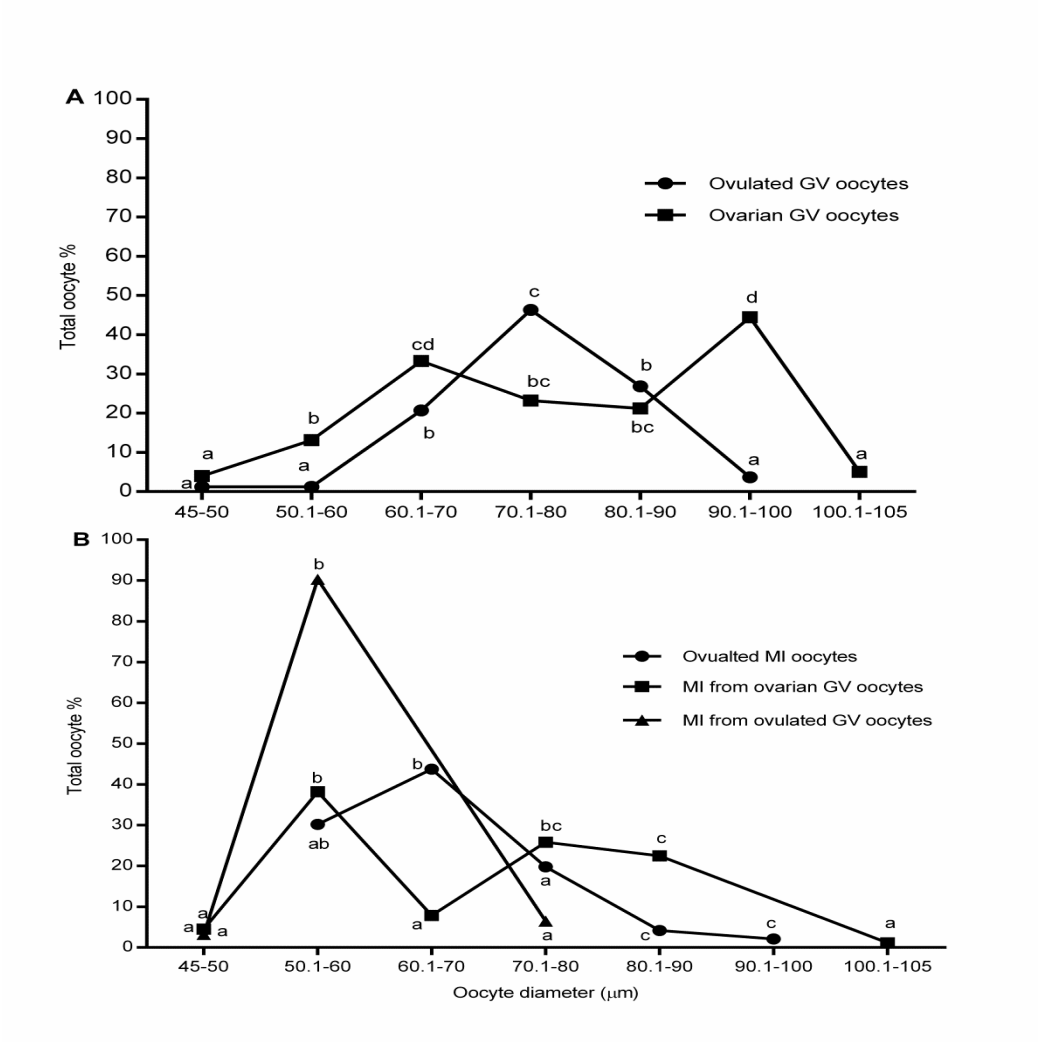

Figure 5.6. Diameter distribution of GV and MI oocytes obtained from different protocols. A: ovarian GV oocytes collected from antral follicles had a wider diameter size range of 50-100μm than ovulated GV oocytes that had a diameter size range of 60-90μm. B: note the uniform diameter size of ovulated MI oocytes of 50-60μm when related to MI oocytes obtained from IVM of ovulated GV oocytes (50-80μm) and ovarian GV oocytes (50-90μm). Data points with different superscripts are significantly different along each curve line ($P<0.05$).

observed with sizes of 60.1-70μm, which in turn was significantly different from oocyte diameter ranges from 45-60μm and 100.1-110μm ($P<0.002$). More

oocytes with size range 50.1-60μm were counted than oocytes with sizes of 45-50μm and 100.1-110μm (13.1% *vs*. 4% and 5%, P<0.05). These data indicate that the ovulated GV oocytes were within a narrower size range (60-90μm) than ovarian GV oocytes, which showed a wider size range of 50-100μM. Also, it is suggested that oocytes of small size of 45-60μm may be found in antral follicles since ovulated oocytes of similar size range were also found but with much lower frequencies (17.1% and 2.5%, respectively, P=0.001, Fig. 5.6A).

Ovulated MI oocytes were found to be within the size range of 50-80μm followed by other size ranges (P<0.002) whereas MI oocytes resulting from IVM of ovulated GV oocytes showed mainly sizes within 50.1-60μm followed by other size ranges (P<0.0001, Fig. 5.6B). Metaphase I oocytes resulting from IVM of ovarian GV oocytes were more frequent within size ranges of 50.1-60μm and 70.1-90μm than other size ranges (P<0.002). These data revealed that while the ovulated MI oocytes and MI oocytes resulting from IVM of ovulated GV oocytes are more abundant within a continuous and narrow size range of 50.1-80μm or 50.1-60μm, respectively, the MI oocytes resulting from IVM of ovarian GV oocytes displayed a high number of oocytes within a non-continuous size range of 50.1-60μm and then 70.1-90μm (Fig. 5.6B).

Although ovulated GV oocytes with small diameters of 45-60μm were detected but with lower rates than that in ovarian GV oocytes collected from the antral follicles, the overall comparison between ovulated and ovarian GV oocyte sizes did not include oocytes of less than 60μm in diameter to exclude any

possible negative impact of the small sizes of oocytes on oocyte quality from either source. Overall, GV oocytes whether ovulated or ovarian were observed to have considerably greater diameter than MI oocytes whether ovulated or obtained from IVM of ovulated or ovarian GV oocytes ($P<0.007$, Fig. 5.7A). This indicates that initiation of meiosis is associated with reduction of oocyte size. Moreover, the ovarian GV oocytes were found to have greater diameters than ovulated GV oocytes (82.02±12.6 *vs*. 76.7±8.26µm, mean±SD, $P<0.007$), which may refer to the readiness of the ovulated GV oocytes to undergo GVBD and to progress to the MI stage, especially, ovulated GV oocytes had higher rates of meiotic development within a shorter duration of time in comparison to ovarian GV oocytes (Table 5.3). The same observation was noted between MI oocytes obtained from IVM of ovulated GV oocytes and other MI oocyte types, which may be attributed to the same reason. Also, we observed that the diameters of GV nuclei in ovulated GV oocytes were smaller than those in ovarian GV oocytes (27.42±3.62 *vs*. 30.27±10.26µm, mean±SD, $P=0.007$, Fig. 5.7B), which may also indicate GV oocytes that had started to undergo GVBD. Conversely, the nucleolus diameters in ovulated GV oocytes were found to be greater than those in ovarian GV oocytes (10.74±0.83 *vs*. 10.21±1.36µm, mean±SD, $P=0.001$, Fig. 5.7C).

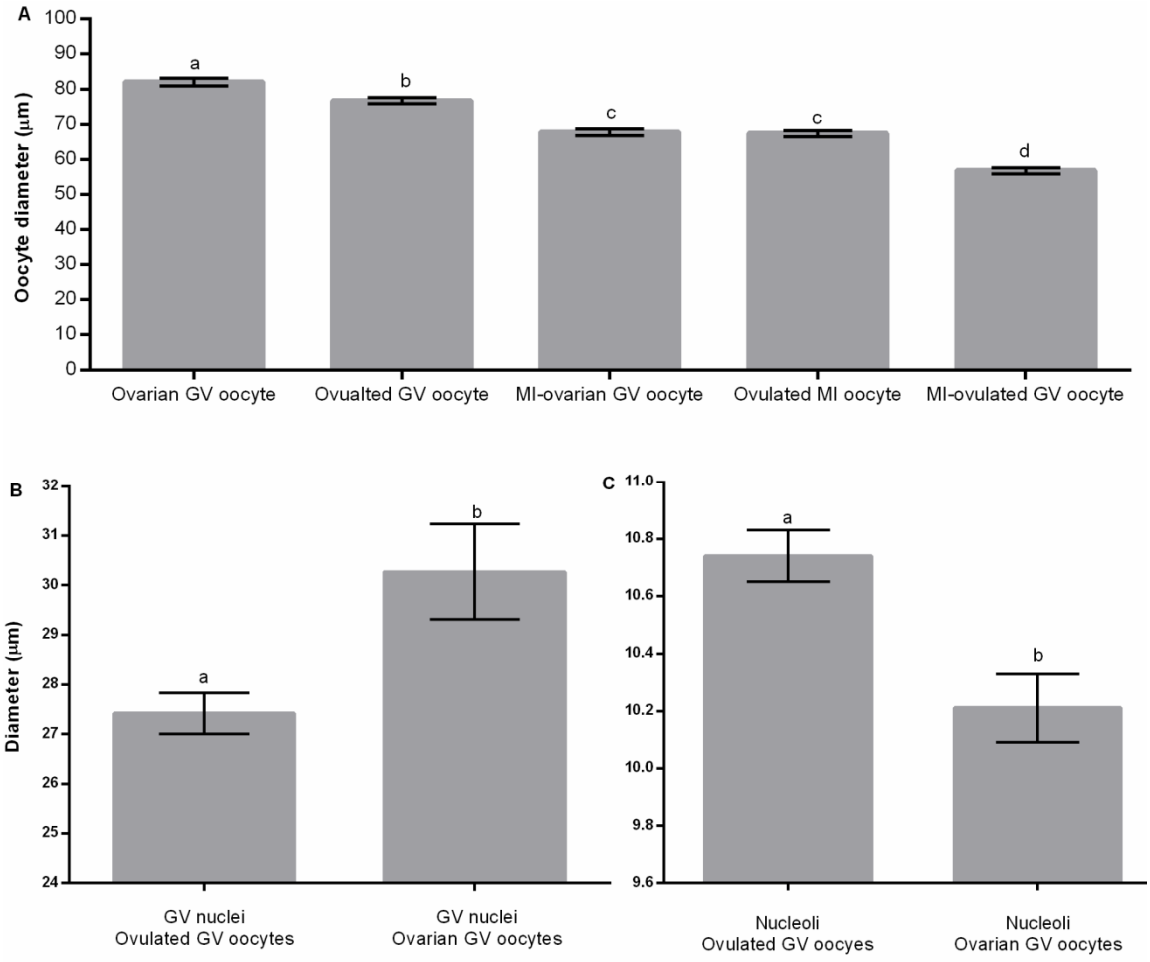

Figure 5.7. Oocyte, GV, and nucleolus diameters of different oocyte types. A: Diameters of oocytes of different origins and meiotic stages. Data points with different superscripts are significantly different (P<0.007). B: GV diameters of ovulated and ovarian GV oocytes (P=0.007). Nucleolus diameters of ovulated and ovarian GV oocytes (P=0.001).

The distributions of oocyte, GV, and nucleolus diameters of ovarian and ovulated GV oocytes among different chromatin configurations were studied. We noted that the diameter of ovarian GV oocytes started to decrease significantly when they displayed SN configuration in comparison to PSN and NSN

configurations (70.12 *vs*. 87.52 and 84.52µm, respectively, P<0.0001, Fig. 5.8) whereas there was no significant difference in the diameter of ovulated GV oocytes displaying SN or PSN configurations (75.3 and 79.11µm, respectively). Only three ovulated GV oocytes showed a NSN configuration, which were considered not representative to be considered in this comparison. Collecting higher numbers of ovulated GV oocytes with NSN configuration might confirm a similar trend to that observed in ovarian GV oocytes, if they display greater diameters. Interestingly, those three ovulated GV oocytes with NSN configuration showed greater diameter of 83.53±4.15µm (mean±SEM).

The GV nuclei of ovarian oocytes among the different configurations did not vary but ovulated GV oocytes of PSN configuration had greater diameters of GV nuclei than those of the SN configuration (29.15 *vs*. 26.64µm, P=0.007). This size reduction in nuclei of the ovulated SN oocytes may relate to the readiness of GV nuclei in the ovulated GV oocytes to undergo GVBD as confirmed by the short duration required for GVBD (Table 5.3). Finally, nucleolus diameters were not different among different chromatin configurations from both oocyte types, indicating that the nucleolus size of GV oocytes does not depend on different chromatin configurations.

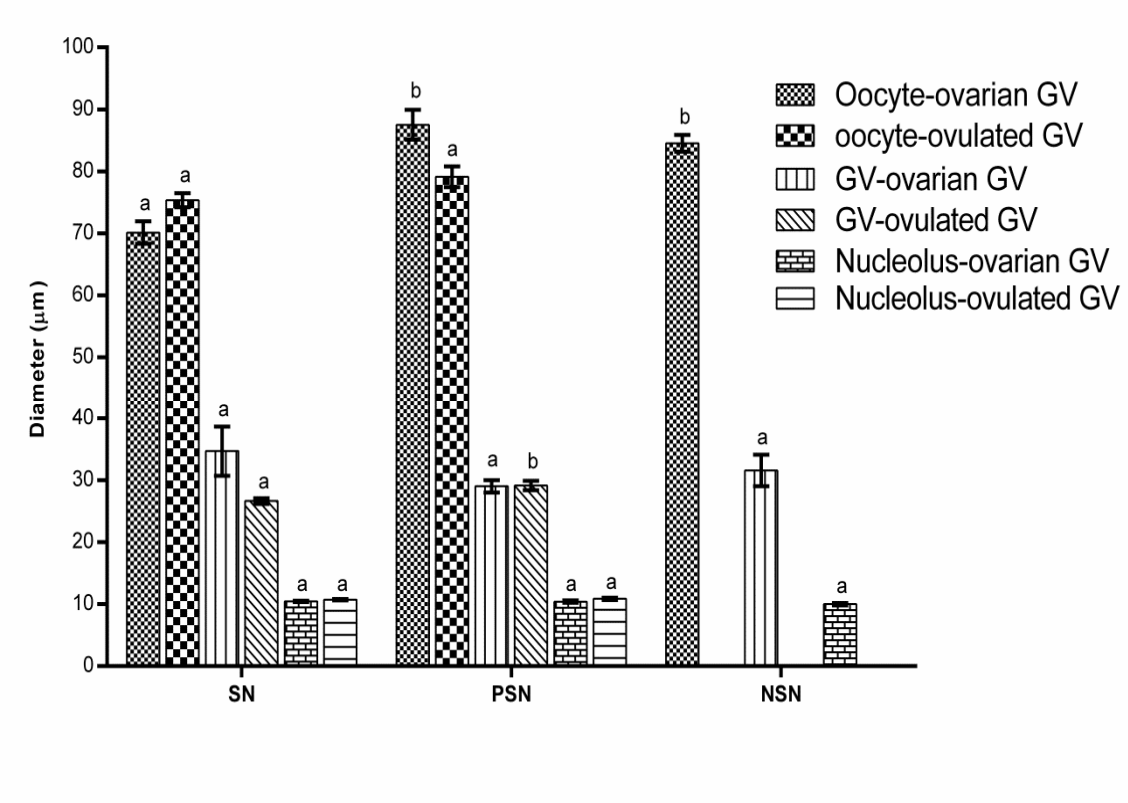

Figure 5.8. Oocyte, GV, and nucleolus sizes of ovulated and ovarian GV oocytes of different chromatin configurations. Different superscripts indicate significant differences among the different chromatin configurations. Data are presented as mean±SEM.

Discussion

The purpose of the present study was to evaluate the quality of ovulated immature oocytes and their potential use as alternatives to ovarian immature oocytes. We observed in this study that ovulated GV oocytes have advanced meiotic and cytoplasmic maturations when compared to ovarian GV oocytes. The advanced meiotic maturation of ovulated GV oocytes was evidenced by a high rate of chromatin configuration of SN type and short duration of GVBD (less

than 1h). The advanced cytoplasmic maturation of the ovulated GV oocytes evoked a high incidence of peripheral CGID, in addition to the continuous and peripheral CG. Such ovulated GV oocytes of SN configuration and peripheral CG with or without intermittent domain correlated with higher rates of GVBD, MI, MII, and IVF when compared to ovarian GV oocytes, which showed mainly NSN configuration and peripheral CG distribution. This state of peripheral CGID precedes the formation of the free domain in the peripheral CG and indicates that the ovulated GV oocytes have started to initiate the free domain in the peripheral CG; a state that is observed in MI oocytes. This further indicates that cytoplasmic maturation, to the extent that CG distribution is a representative indicator of cytoplasmic maturation, is independent from meiotic maturation and that it can precede meiotic maturation when ovulation of GV oocytes is induced. On the other hand, ovulated MI oocytes and MI oocytes obtained from IVM of ovulated GV oocytes showed higher rates of normal spindles with chromosomes assembled at the metaphase plates than those of MI oocytes obtained from IVM of ovarian GV oocytes, indicating that ovulated MI and MI oocytes from ovulated GV oocytes have higher quality. Moreover, the CG distribution between ovulated MI oocytes and MI resulting from ovarian GV oocytes were similar except with a considerable increase of exocytosed CGs in the ovulated MI oocytes. We observed a reduction in oocyte size upon initiation of meiotic maturation and ovulation. Also, ovulated immature oocytes have convenient features such as ease of retrieval, reduced time for retrieval, high yield, and being representative

of the cohort of ovarian follicles that ultimately undergo ovulation. It was also noted that ovulated immature oocytes have more uniform sizes when compared to ovarian oocytes retrieved from the antral follicles, suggesting that oocytes collected from antral follicles do not always represent oocytes that would be ovulated.

Many factors such as oocyte/follicle size and age have been found to influence chromatin configuration of oocytes (Scharmm *et al*. 1993; Zuccotti *et al*. 1995). In this study, ovulated GV oocytes were influenced by PMSG, hCG, and CLZ; and consequently with changes in the cAMP and GJC levels. Mice primed with PMSG were found to increase GV oocytes with SN configuration (Mattson and Albertini 1990; Tan *et al*. 2009; Zuccotti *et al*. 1995). The IVM of such oocytes showed higher meiotic maturation and competence with a lower level of autoactivation than GV oocytes of NSN configuration (Zuccotti *et al*. 1998 and 2002). Higher proportions of ovarian GV oocytes of bitches had grouped chromatin (comparable to SN) when isolated after the LH peak than before the LH peak (Reynaud *et al*. 2009). Similarly, the SN:NSN ratio of ovarian GV oocytes in mice increased after hCG-induced ovulation (Bouniol Baly *et al*. 1999). These data indicate that NSN immature oocytes are not ready to be ovulated and need to reach the SN configuration. Moreover, it was suggested that the SN oocytes that failed to ovulate had undergone atresia. This is further confirmed with a high proportion of SN oocytes in aged mice. On the other hand, it is speculated for NSN oocytes that fail to reach SN configuration are to be

recruited following the LH surge (Tan *et al. 2009;* Zuccotti *et al.* 1998 and 2005). In addition to the gonadotropin effects, active GJC between oocytes and follicular cells are believed to maintain high levels of intraoocyte cAMP. Such a state was found to be associated with oocytes with low meiotic development and NSN configuration (Lodde *et al*. 2007). Conversely, interrupting the GJC was found to condense the chromatin and cease the RNA synthesis (Luciano *et al*. 2011). On the other hand, hCG/LH is known to disrupt GJC, decrease oocyte cAMP, and induce SN configuration (Bouniol Baly *et al*. 1999; Hashimoto *et al*. 1988; Isobe *et al*. 1998). In regard to PDE3-I effects on chromatin configurations, arresting oocytes at the GV stage via a PDE3-I was found not only to increase intraoocyte cAMP levels but also SN configuration of oocytes (Nogueira *et al*. 2003a; Vanhoutte *et al*. 2007). These observations collectively indicate that inhibition of GJC reduces cAMP outflow from the granulosa cells to oocytes and increases the tendency of chromatin to organize in the SN configuration. Moreover, arresting oocytes at the GV stage via a PDE3-I elevates intraoocyte cAMP and enhances SN configuration of oocytes. It is suggested that follicular cAMP, when having active GJC, causes oocyte chromatin to have NSN configuration, and when intraoocyte cAMP increases, results in SN configuration. This is further supported by our previous and present results that showed that ovulated GV oocytes, which lack GJC and have moderate cAMP levels, have a SN configuration whereas ovarian GV oocytes, which are surrounded by compact cumulus cells and have high cAMP levels,

have NSN configuration (Chapter III). We are suggesting the following scenario for the high incidence of SN with ovulated GV oocytes and NSN configuration with ovarian GV oocytes: priming mice with PMSG increased oocytes with SN configuration. This is further potentiated by hCG administration that disrupts GJC and reduces cAMP flow to oocytes. Finally, administration of CLZ inhibits PDE3, arrests oocytes at GV, and increases the intraoocyte cAMP, which exacerbates the SN occurrence in ovulated GV oocytes. Those factors collectively aid to synchronize GV oocytes at SN configuration and consequently increase meiotic and developmental competence.

The distribution of CG has been used as a criterion to evaluate cytoplasmic maturation in addition to mitochondrial distribution and cytosolic redox state (Curnow *et al*. 2010; Dumollard *et al*. 2007; Liu *et al*. 2005; Miao *et al*. 2009; Miyara *et al*. 2003; Sha *et al*. 2010,). Liu and coworkers suggested a role for MI chromosomes to induce free domain in the peripheral CG because they found MI stage preceded the initiation of free domain and no peripheral free domain was observed in GV oocytes arrested with PDE3-I up to 15h *in vitro* (Liu *et al*. 2005). Here, we observed CGID in ovulated GV oocytes indicating that formation of peripheral CGFD may not be dependent of meiotic maturation. Similar to chromatin configuration, the CG in our ovulated GV oocytes were exposed to CLZ and gonadotropins and their consequences. An increase of gonadotropin concentrations in the culture medium resulted in an increase in CG migration toward the periphery (Sha *et al*. 2010). Collection of oocytes post-hCG

in superovulated mice showed shorter durations of time required to form 1st or 2nd free domain in the peripheral CG in comparison to oocytes matured *in vitro* (Liu *et al*. 2005). Moreover, Stoker and coworkers showed that delaying ovulation in rats by one day via suppression of the LH surge showed abnormal CG patterns (Stoker *et al*. 2003). The latter study showed the essential role of the ovulatory stimulus on CG distribution as an abnormal CG pattern was observed when follicular fully grown GV oocytes had delayed GVBD due to lack of LH effect. In the present study, oocytes were arrested at the GV stage without interfering with hCG/LH effects and were showing advanced stages of distribution of CG in comparison to ovarian GV oocytes. These data indicate that LH/hCG aids to control CG distribution and may be the factor for the formation of the intermittent domain in ovulated GV oocytes. In regard to the effect of PDE3 inhibition on CG distribution, Liu and colleagues showed that the arrest of oocytes with hypoxanthine allowed CGs to align at the periphery in only a portion of the oocytes when incubated up to 15h, in agreement with previous reports (Duque *et al*. 2002; Liu *et al*. 2005; Nogueira *et al*. 2003b). This suggests a role for elevated cAMP that does not support CG migration. These data along with our observations lead us to conclude that CG distribution is controlled by the ovulatory stimulus that overcomes the inhibitory role of cAMP on CG distribution. Furthermore, both advanced cytoplasmic and meiotic maturation, as evidenced by peripheral CGID and SN configuration, respectively, can result in ovulated GV oocytes undergoing GVBD within an hour, mature into MII stage

with high yield and within 10h, and associate with high IVF rates. On the other hand, ovarian GV oocytes that had peripheral CG distribution in addition to dominant NSN configuration require approximately 3h for GVBD to be completed in all oocytes and matured into MII stage in low yield and within 14h. These ovarian GV oocytes were associated with low IVF rates when compared to ovulated GV oocytes.

Interestingly, some of the ovulated MI oocytes showed CG activation. We suggest that those oocytes had undergone a premature exocytosis. Exocytosis of CG is observed in aged, activated, or fertilized MII oocytes (Liu *et al*. 2003; Miao *et al*. 2009; Xu *et al*. 1997). Although the ovulated MI oocytes from mice treated with 7.5mg CLZ at the same time as hCG were found to have exocytosed CG (as reported in this study), another study showed the IVF rate of ovulated MI oocytes, resulting from animals treated with 7.5mg CLZ at 4 or 7h pre-hCG, was higher than that of ovulated MII oocytes from non-treated mice (Taiyeb-Ridha *et al*. 2011b). It is suggested that the premature exocytosed CGs in ovulated MI oocytes obtained from mice treated with 7.5mg CLZ at the same time as hCG does not influence the IVF rate because only 11.6% of MI oocytes showed exocytosed CG counterbalanced by a high rate of organized spindle and chromosomes and potential positive effect of MI oocyte uniform size. It is also suggested that ovulated MI oocytes from mice treated at 4h or 7h pre-hCG with CLZ have low levels of premature exocytosed CG and consequently higher IVF rates.

It was observed in this study that the GV oocytes (whether ovulated or ovarian) started to decrease in size when transitioning to MI oocytes probably due to utilization of proteins, substrates, factors, and nutrients in the cytoplasm (Gandolfi and Gandolfi 2001; Watson 2007) and/or due to GVBD and consequent size shrinkage. If MII oocytes are found to be smaller than MI oocytes, the considerable decrease in size of MI oocytes obtained from IVM of ovulated GV oocytes in comparison to other MI oocytes could be attributed to MI oocytes that are closer to emitting the 1st polar body, form a perivitelline space, and/or further utilize factors and proteins in the cytoplasm. This is further supported by the fact that ovulated GV oocytes required 10h to transition to MII oocytes whereas ovarian GV oocytes required a longer period. Moreover, ovulated MI oocytes from mice treated with CLZ at 4 or 7h pre-hCG required 3 or 6h, respectively, to extrude the 1st polar body (Taiyeb-Ridha *et al*. 2011b).

Tartia and colleagues found that the reduction in oocyte size was completed during the MI stage; approximately 10h post-hCG. This reduction in oocyte size is controlled through accumulation of glycine, which provides intracellular osmotic support to control cell volume homeostasis during size reduction. Glycine accumulation was found to be mediated through glycine transporters (GLYT1). Activation of GLYT1 follows the ovulatory stimulus and the initiation of meiotic maturation but was found to be quiescent in freshly isolated GV oocytes. Interestingly, arresting oocytes at the GV stage *in vitro* using cAMP agonist compounds showed both size reductions and activation of

GLYT1, indicating that the GLYTI transporter does not require meiosis or hCG when spontaneous maturation is blocked (Tartia *et al*. 2009). It is hypothesized that ovulated GV oocytes were able to reduce size more than ovarian GV oocytes because ovulated GV oocytes have active CLYT1 due to the hCG trigger, arrest of meiosis induction. Such oocytes are believed to be close to initiation of meiosis as they are characterized by advanced levels of maturation and short duration of GVBD. Ovarian GV oocytes may lack all of those triggers that are required to activate the mechanism that regulates cell volume homeostasis during size change and consequently have greater diameters than ovulated GV oocytes.

As a concept, oocyte maturation and competence were reported to have a positive correlation with oocyte size (Anquita *et al*., 2007, Comizzoli *et al*., 2011; Hirao *et al*., 1993; Otoi *et al*., 2000; Raghu *et al*., 2002). Here, we observed that ovarian and ovulated GV oocytes reduce their size upon meiosis. Therefore, we add to this concept additional information, that is, although oocyte competence is positively related to oocyte size, immature oocytes will have reduced their size upon meiosis. Also, it is suggested that relatively smaller immature oocytes collected from ovaries after an ovulatory stimulus could mean collection of oocytes with advanced developmental competence when compared to greater immature oocytes collected from ovaries after the stimulus. This is supported by the fact that ovulated GV oocytes revealed an advanced meiotic and cytoplasmic development and higher fertilization rate and were smaller in

size when compared to the ovarian GV oocytes that were greater in sizes and of lower meiotic and cytoplasmic maturation and fertilization rates. Therefore, the decrease in diameter of GV oocytes can be considered as an additional parameter to consider when evaluating developmental maturation and competence.

Normality of spindle morphology and chromosome alignment at the spindle equator have been used as indicators to assess oocyte quality and capability to form chromosomally balanced embryos. Arresting ovarian oocytes at the GV stage using PDE3-Is followed by IVM not only maintained normal morphology of spindles and alignment of chromosomes at the metaphase plates but also synchronized and improved cytoplasmic and meiotic maturation and elevated oocyte competence (Albuz *et al*. 2010 Nogueira *et al*. 2003a and 2005b; Vanhoutte *et al*. 2007 and 2008). Here, we extend the beneficial effect of temporal arrest of oocytes at the GV stage *in vitro* to the *in vivo* level and have found similar results to those reported *in vitro*. Although the spindle length did not differ among different MI oocytes, the spindle width of MI oocytes obtained from IVM of ovarian GV oocytes was greater. Sanfins *et al.* indicated that *in vivo* matured oocytes from naturally ovulated mice showed a compact spindle with focused poles whereas the *in vitro* matured MI oocytes exhibited a barrel-shaped spindle with flat poles (Sanfins *et al*. 2003). The observed wider spindle in our MI oocytes obtained from IVM of ovarian GV oocytes may indicate the same phenomenon as oocytes with barreled spindles.

In summary, adding to our previous work in which we presented protocols for retrieval of ovulated oocytes at different stages of meiotic maturation, we here evaluated meiotic and cytoplasmic maturations and fertilization of ovulated immature oocytes. We observed that ovulated immature oocytes are at advanced meiotic and cytoplasmic maturation levels with organized spindles and chromosomes. We also found that GV oocytes are larger than MI oocytes and the ovulated immature oocytes are smaller than their ovarian counterparts. We therefore suggest that although oocytes of large volumes are correlated with high developmental and competence rates, they reduce size as meiosis is triggered.

Summary and conclusion

- Administration of CLZ in superovulated mice results in ovulation of GV oocytes with advanced chromatin configuration (SN) when compared to ovarian GV oocytes resulting from hyperstimulated mice.
- Administration of CLZ in superovulated mice resulted in ovulation of GV oocytes with advanced cytoplasmic maturation as evidenced by the initiation of an intermittent domain of CG instead of the continuous peripheral CG noted in ovarian GV oocytes collected from PMSG primed mice.
- Ovulated GV oocytes have an advanced meiotic maturation that is merged with their cytoplasmic maturation because SN configuration

occurs with both peripheral CG with or without intermittent domain whereas no such a trend occurs with ovarian GV oocytes.

- Ovulated GV oocytes have higher rates of maturation and fertilization than ovarian GV oocytes.
- Ovulated GV oocytes can resume meiosis with shorter periods of time than ovarian GV oocytes.
- Ovulated MI oocytes and MI oocytes resulting from IVM of ovulated GV oocytes have higher quality (normal spindle and chromosomal organization) than ovarian MI oocytes collected from IVM of ovarian GV oocytes.
- MI oocytes collected from IVM of ovarian GV oocytes have wider spindles (a state that occurs in in-vitro matured oocytes more than *in vivo* matured oocytes) than ovulated MI oocytes and MI oocytes obtained from IVM of ovulated GV oocytes.
- Ovulated MI oocytes resulting from administration of CLZ at the same time as hCG have significant rates of premature exocytosis of CG.
- Ovulated GV oocytes are more uniform in diameter than ovarian GV oocytes.
- *In vitro* matured ovulated GV oocytes have the most uniform size of diameters followed by ovulated MI oocytes and finally MI oocytes resulting from IVM of ovarian GV oocytes.

- Meiosis is associated with size reduction because MI oocytes are smaller than GV oocytes, and ovulated GV oocytes that have advanced meiotic and cytoplasmic maturation and consequently faster and higher rate of maturation and meiosis are smaller than ovarian GV oocytes.
- Ovulated GV oocytes have smaller GV nuclei than ovarian GV oocytes.
- Ovulated GV oocytes have greater nucleoli than ovarian GV oocytes.

CHAPTER VI

SYNCHRONIZATION OF OOCYTE MATURATION IN SUPEROVULATED MICE IMPROVES IN VITRO FERTILIZATION RATE

Overview

In vitro synchronization of oocyte nuclear and cytoplasmic maturation has been found to improve the IVF rate of *in vitro* matured ovarian oocytes in comparison with non-synchronized corresponding oocytes. Here, we tested the hypothesis that synchronization of oocyte maturation in superovulated mice using CLZ would increase IVF rates when compared to non-synchronized superovulated oocytes. Mice were superovulated (control) to result in ovulation of MII oocytes whereas treated mice were superovulated and treated with CLZ to result in ovulation of GV or MI oocytes. Ovulated control oocytes were *in vitro* fertilized directly upon collection whereas ovulated MI or GV oocytes were *in vitro* matured followed by IVF. Oocytes from treated mice were collected later to allow the ovulated GV or MI oocytes to mature in the oviduct followed by IVF. All ovulated MI oocytes that were *in vitro* or *in vivo* matured showed higher IVF rates than control oocytes. Only ovulated GV oocytes that were *in vitro* matured showed high IVF rates whereas the *in vivo* matured showed low IVF rates in comparison to control oocytes. It is concluded that temporal arrest of oocyte maturation during superovulation can increase the IVF rate in mice.

Introduction

The term oocyte cytoplasmic maturation refers to events that occur during two distinctive phases of oocyte development. The first phase occurs during follicle growth and the second phase occurs during meiotic maturation of the fully grown oocyte. Oocyte cytoplasmic maturation during follicle growth includes accumulation and storage of maternal mRNA, proteins, substrates, nutrients, factors, and their regulatory processes (Gandolfi and Gandofi 2001; Krisher 2004; Watson 2007). This cytoplasmic maturation phase is essential for early embryonic survival, especially before maternal-zygotic gene transition, which is around the 2 and 4/8-cell stages in mouse and human embryos, respectively. Until embryonic genome activation, the embryo continues to rely on the maternal reserve that was produced during cytoplasmic maturation in the growing follicle follicle (Bettegowda and Smith 2007; Minami *et al*. 2007). The higher developmental capacity observed in large oocytes compared to small oocytes in many species suggests more accumulation of nutrients and transcripts, and consequently larger oocyte sizes that efficiently support early embryonic development (Marchal *et al*. 2002; Otoi *et al*. 2000 and 2001; Raghu *et al*. 2002; Schramm *et al*. 1993). This cytoplasmic maturation was found to cease as the fully grown oocyte of prophase I started to enter meiotic maturation as usually evidenced by the lack of fluorescent transcriptional activity around the nucleolus of GV oocytes (Bounio-Baly *et al*. 1999; De La Fuente and Eppig 2001; Tan *et al*. 2009). In addition to the role of the oogenic cytoplasmic maturation in

fostering early embryonic development, it is also associated with the development of meiosis and embryonic progression post-zygotic gene activation (Gandolfi and Gandolfi 2001; Krisher 2004; Minami *et al*. 2007; Renard *et al*. 1994; Watson 2007).

Oocyte cytoplasmic maturation during meiotic resumption includes some morphological and biochemical events such as CG migration, microfilament relocation, MAP kinase phosphorylation, cyclin B synthesis, and $p34^{cdc2}$ kinase activation. The CG distribution was found to migrate to the cortex of oocytes to regulate fertilization and prevent polyspermy after fertilization (Barros and Yanagimachi 1971; Liu *et al*. 2005) whereas microfilaments control nuclear and CG movement, meiotic spindle rotation, and others (Sun and Schatten 2006). Cyclin B, in addition to other kinases and phosphatases, are important for $p34^{cdk2}$ to gain its protein kinase activity (Doree and Hunt 2002; Marangos and Carroll 2004; Sun *et al*. 2001) whereas $p34^{cdc2}$ kinase activity is essential to GV oocyte transition into the MI stage and then to sustain MII arrest until fertilization (Choi *et al*. 1991; Hampl and Eppig 1995). Moreover, MAP kinase was also found to associate with arresting oocytes at the MII stage as c-mos (MAP kinase kinase kinase) knockout mice resulted in MII oocytes undergoing spontaneous parthenogenesis and/or third metaphase (MIII) stage (Carlton 1994, Jones 2004, Verlhac *et al*. 1996).

The temporal arrest of oocyte meiotic maturation *in vitro* was reported to increase the oocyte cytoplasmic maturation and quality, and consequently IVF

and blastocyst rates in several species when compared to non-arrested ovarian GV oocytes undergoing spontaneous IVM (Albuz *et al*. 2010; Nogueira *et al*. 2003a and 2005a; Shu *et al*. 2008; Vanhoutte *et al*. 2008 and 2007). The observed high rates of IVF and blastocysts never exceeded that of ovulated MII oocytes. Previously, we found administration of CLZ *in vivo* resulted in ovulation of immature oocytes that have more advanced cytoplasmic and meiotic maturation when compared to *in vitro* matured oocytes that were not arrested (Chapter V). Here, we tested the capability of such ovulated immature oocytes to resume meiotic maturation *in vitro* or *in vivo*. Also, we monitored the *in vitro* and in vivo course of maturation time of these oocytes. We then *in vitro* fertilized those oocytes and observed their 2-cell embryo rates and compared them with those resulting from ovulated mature oocytes using a superovulated mouse model.

Materials and methods

Mice and superovulation

Swiss Webster mice (8-10 weeks old) were ip injected with 10 IU PMSG at 6:00pm followed in 47h with 10 IU hCG. All CLZ doses were dissolved in aliquots of 0.1ml DMSO just before administration. Control mice received 0.1ml DMSO at 7h pre-hCG, 4h pre-hCG, or at 4h pre-hCG and 2h post-hCG. Ovulated oocytes from control mice were retrieved 14h post-hCG and underwent IVF directly. Ovulated oocytes from treated mice that underwent IVM or oviductal

maturation and IVF were incubated in HTF medium supplemented with 4.5% FBS at 37°C with 5% CO_2in humidified air. Oocyte and spermatozoa collection media were HTF supplemented with HEPES and 4.5% FBS. All gonadotropins were purchased from Intervet Inc. (Summit, NJ) whereas the culture media were obtained from Zenith Biotech (Guilford, CT). CLZ was purchased from LKT Laboratories (St. Paul, MN) and all experiments were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Experimental design

Experiments were conducted to test the following hypotheses: 1) Ovulation of MI oocytes in superovulated mice treated with 7.5mg CLZ, 7 or 4h pre-hCG, followed by maturation for 3 or 6h, respectively, *in vitro* or *in vivo* yields IVF rates that are higher than those observed in ovulated oocytes collected from superovulated mice not treated with CLZ. 2) Ovulation of GV oocytes in superovulated mice treated with 7.5mg CLZ, 4h pre-hCG and 2h post-hCG, followed by IVM or *in vivo* maturation (IVOM) for 10h yields IVF rates that are higher than those observed in ovulated oocytes collected from superovulated mice not treated with CLZ. Figure 6.1 shows the experimental design for testing these hypotheses.

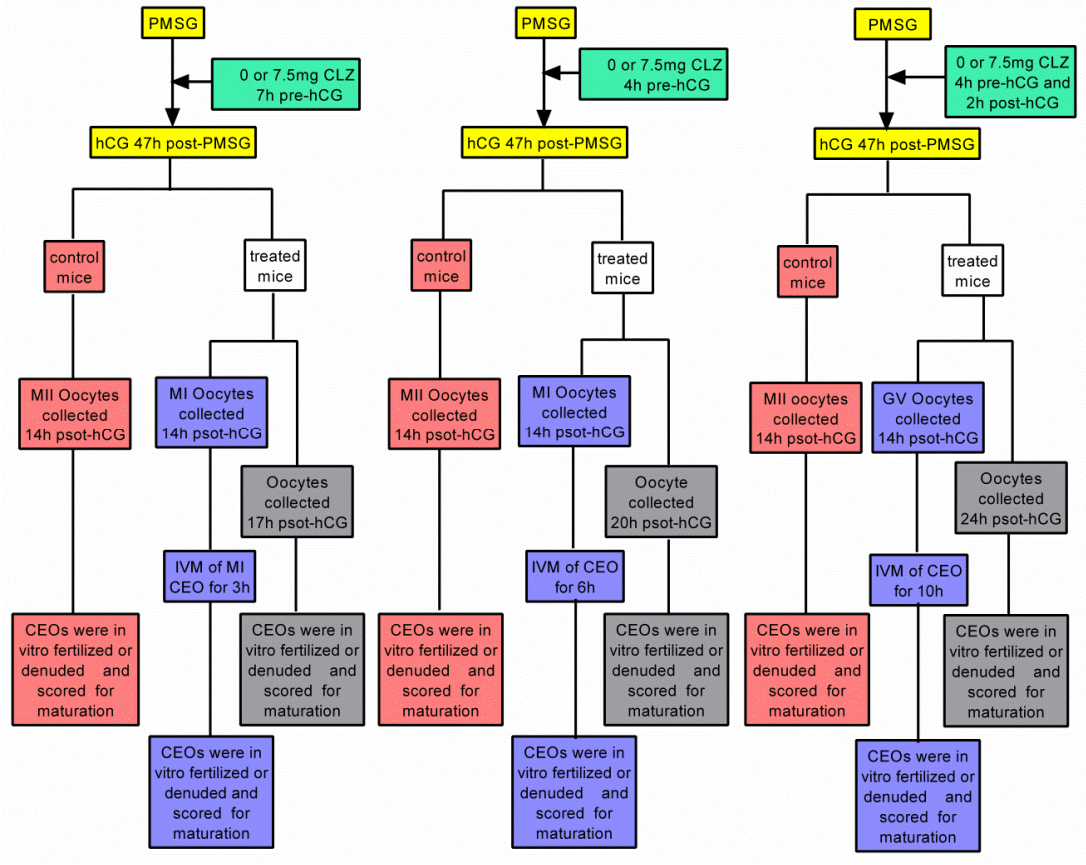

Figure 6.1. The experimental design of chapter six. Ovulated germinal vesicle (GV) and metaphase I (MI) oocytes were *in vitro* or *in vivo* matured (IVM or IVOM, respectively) to mature oocytes followed by *in vitro* fertilization (IVF) from superovulated mice; pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (47h apart). Cilostazol (CLZ) was administered in the treated group at the designated times.

Mice treated with 7.5mg CLZ at 7h or 4h pre-hCG

To study the capability of ovulated MI oocytes to undergo *in vitro* or *in vivo* (in the oviduct) maturation and IVF, mice were treated with 7.5mg CLZ, 7h pre-hCG, and were divided into two groups. For the first group, oocytes were retrieved 14h post-hCG (8:00am) and then *in vitro* matured for 3h (until

11:00am). For the second group, oocytes were retrieved 17h post-hCG (11:00am). Oocytes from both groups were denuded in 0.006% hyaluronidase for 2 minutes and scored for their maturational status using a Nikon SMZ1500 stereomicroscope and an Olympus IX71 inverted microscope. In the following experiments, the superovulated treated mice were sacrificed and the ovulated CEO underwent IVM or IVOM as described followed by IVF (using the same semen suspension) without denudation at 11:00am. All oocytes were then incubated for 1 day and scored for the 2-cell embryo endpoint.

Other mice were treated with 7.5mg CLZ, 4h pre-hCG, and were divided into two groups. Ovulated oocytes from the first group were retrieved 14h post-hCG and *in vitro* matured for 6h (up to 2:00pm). For the second group, oocytes were retrieved 20h post-hCG (2:00pm). Oocytes from both groups were then denuded and scored for their maturational status or underwent IVF without denudation as described above.

Mice treated with 7.5mg CLZ at 4h pre-hCG and 2h post-hCG

To study the capability of ovulated GV oocytes to undergo *in vitro* or *in vivo* maturation and IVF, mice were treated with 7.5mg CLZ, 4h pre-hCG and 2h post-hCG, and were divided into two groups. For the first group, oocytes were retrieved 14h post-hCG and *in vitro* matured for 10h (up to 6:00pm). For the second group, oocytes were retrieved 24h post-hCG (6:00pm). All oocytes were then denuded and scored for their maturational status. In another experiment,

the superovulated treated mice were sacrificed and ovulated oocytes were collected and underwent IVM or IVOM as described, followed by IVF (using the same semen sample) without denudation at 6:00pm. All oocytes were scored 1 day after IVF for the 2-cell embryo endpoint.

In vitro *fertilization*

The tails of the epidiymides attached to the ductus deferens were placed in 1.5ml HTF medium with HEPES. The ductus deferens attached to the posterior thick tubule of the epididymis was separated followed by flushing with the HTF medium using a 26 gauge blunted needle. A few cuts were made in the remaining epididymis tail to allow more spermatozoa to swim out. The spermatozoa suspension was incubated for 10-15 minutes for capacitation. Concentrations of 2 million spermatozoa/ml were prepared in the medium containing oocytes collected from three females. Oocytes were incubated with spermatozoa for 6h followed by three washes and incubated for one day.

Results

We previously showed that CLZ administered 7 or 4h pre-hCG in superovulated mice resulted in ovulation of more than 90% MI oocytes. Moreover, we observed that the ovulated MI oocytes resulting mice treated with 7.5mg CLZ, 7h pre-hCG, were semi-MII oocytes as evidences by the presence of membranous protrusions indicating the imminent extrusion of the 1st polar body (Chapter III).

Here, we found that delaying retrieval of such ovulated MI oocytes (semi-MII oocytes) to 17h post-hCG (11:00am) or retrieving the oocytes 14h post-hCG (8:00am) followed by IVM until 11:00am allowed all of these MI oocytes to finish meiotic maturation with a yield of MII oocytes that was similar to that in control mice. In the following experiments, MII oocytes resulting from IVM or IVOM of those ovulated MI oocytes were *in vitro* fertilized 17h post-hCG without denudation because of the importance of the cumulus cells for fertilization and embryonic development. Both *in vivo* and *in vitro* matured MI oocytes from mice treated with 7.5mg CLZ at 7h pre-hCG showed higher rates of 2 cell embryo production when compared to ovulated MII oocytes from control superovulated mice (90.6% and 88.2% *vs*. 81.5%, $P<0.0001$ and $P=0.017$, respectively, Fig. 6.2).

Scoring of the ovulated MI oocytes 20h post-hCG (2:00pm) from mice treated with 7.5mg CLZ, 4h pre-hCG, showed oocytes at the MII stage whether matured *in vitro* or *in vivo* (in the oviduct). For those oocytes, IVM or IVOM followed by IVF without denudation 20h post-hCG resulted in high rates of 2-cell embryos when compared to the IVF rate observed in ovulated MII oocytes from control mice (89% and 87.8% *vs*. 80.2%, $P=0.007$ and $P=0.014$, respectively, Fig. 6.3).

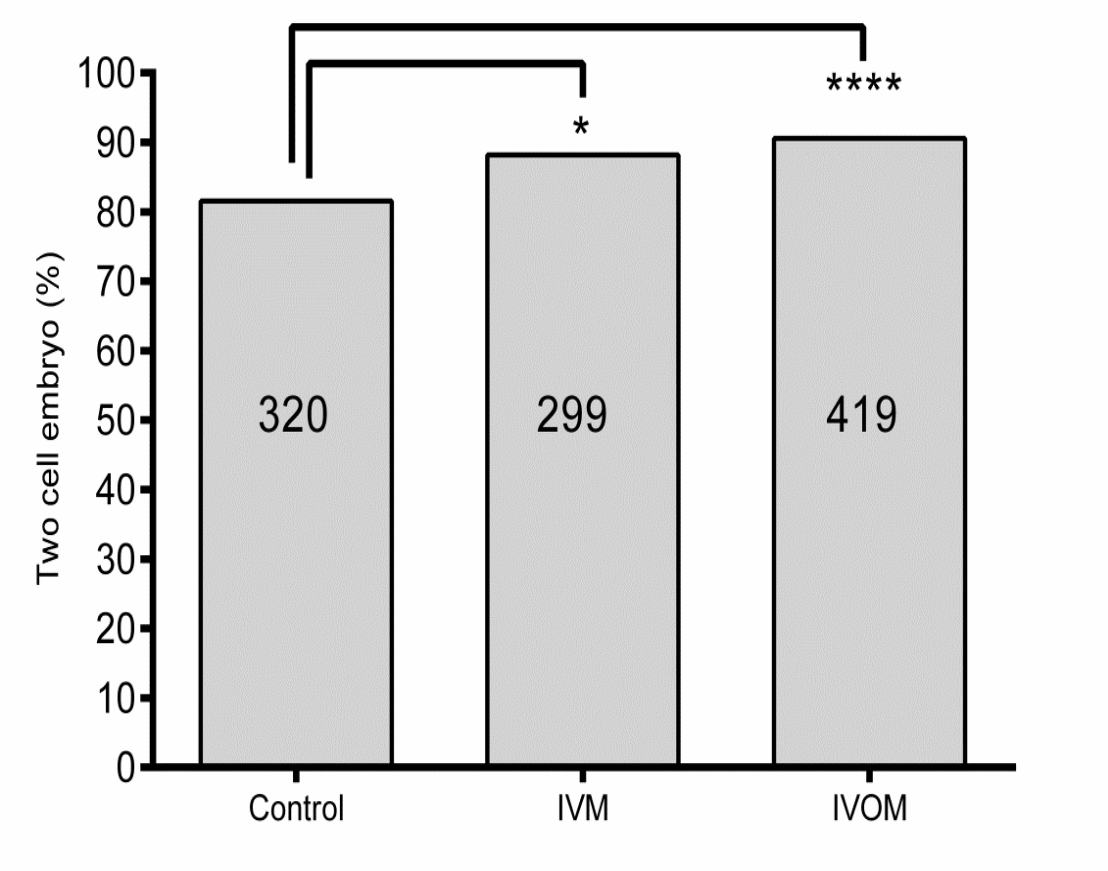

Figure 6.2. Two cell embryo rates for ovulated MI oocytes resulting from Mice treated with 7.5mg CLZ at 7h pre-hCG. Ovulated MI oocytes were collected 14h post-hCG followed by IVM for 3h and IVF or at 17h post-hCG (IVOM) followed by IVF. Oocytes from control mice were collected 14h post-hCG and underwent IVF. IVM: *in vitro* maturation; IVOM: *in vivo* maturation. Number inside each bar is the total number of oocytes evaluated. *<0.1 and ****<0.0001.

Here, we found that scoring of the ovulated GV oocytes that underwent IVM for 10h revealed a yield of MII oocytes that was similar to that observed in control mice. On the other hand, GV oocytes retrieved from oviducts 24h post-hCG (6:00pm) also resulted in a similar yield of MII oocytes to that observed in

control mice but some of those MII oocytes were degenerated; fragmented (P=0.01).

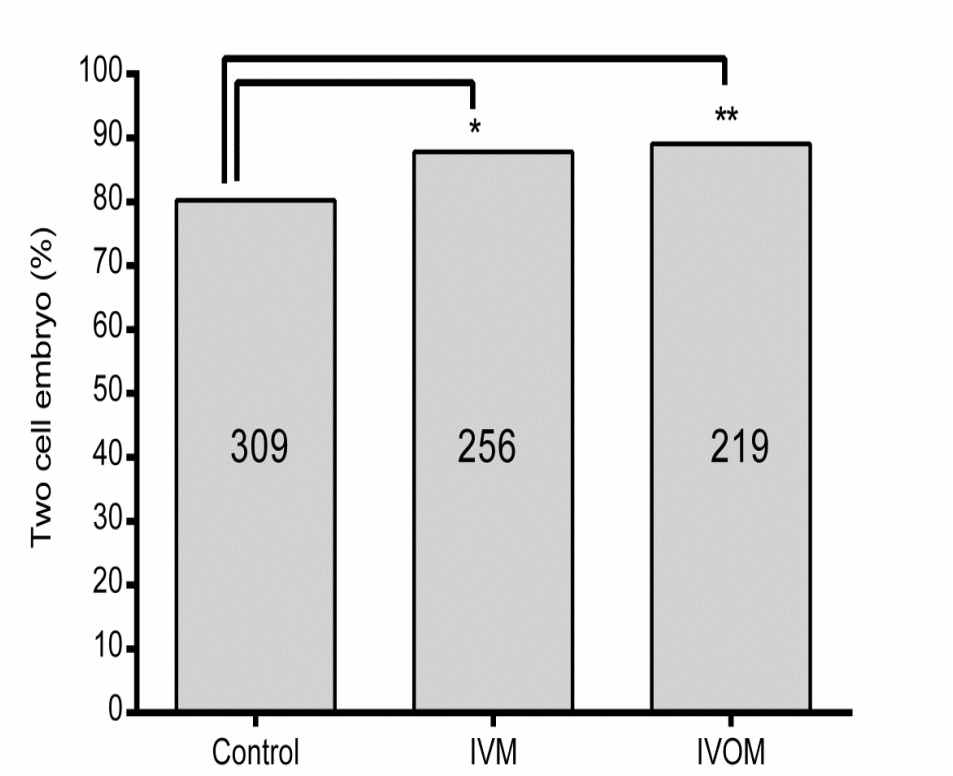

Figure 6.3. Two cell embryo rates for ovulated MI oocytes resulting from Mice treated with 7.5mg CLZ at 4h pre-hCG. Ovulated MI oocytes were collected 14h post-hCG followed by IVM for 6h and IVF or at 20h post-hCG (IVOM) followed by IVF. Oocytes from control mice were collected 14h post-hCG and underwent IVF.IVM: *in vitro* maturation; IVOM: *in vivo* maturation. Number inside each bar is the total number of oocytes evaluated. *<0.05 and **<0.01.

The *in vitro* matured ovulated GV oocytes showed a higher IVF rate than that of control MII oocytes (95.2% *vs*. 89.4%, P=0.013) but the *in vivo* matured (oviductal matured) oocytes had a lower rate of cleavage (73.5%) in comparison

to control MII oocytes and *in vitro* matured GV oocytes (P<0.0001, Fig. 6.4). To exclude the effect of degenerated MII oocytes on this low fertilization rate, the *in vivo* matured GV oocytes were denuded just before IVF, and the degenerated oocytes were removed before IVF. Control ovulated MII oocytes were also denuded before conducting IVF to control for the denudation effect on fertilization. At 24h post-fertilization of the denuded oocytes, the *in vivo* matured

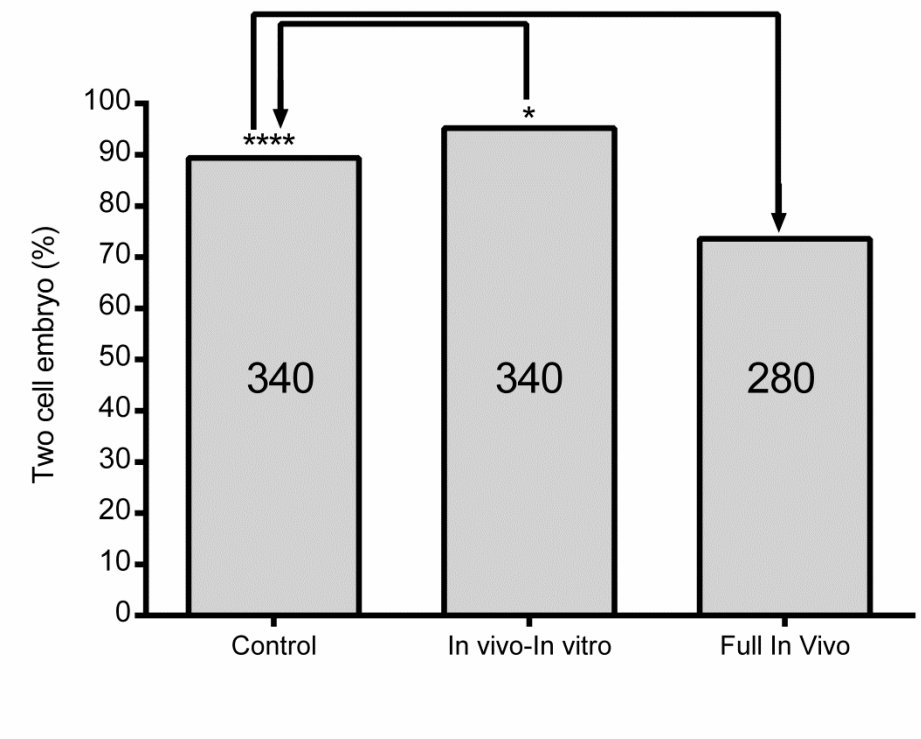

Figure 6.4. Two cell embryo rates for ovulated GV oocytes resulting from Mice treated with 7.5mg CLZ at 4h pre-hCG and 2h post-hCG. Ovulated GV oocytes were collected 14h post-hCG followed by IVM for 10h and IVF or at 24h post-hCG (IVOM) followed by IVF. Oocytes from control mice were collected 14h post-hCG and underwent IVF.IVM: *in vitro* maturation; IVOM: *in vivo* maturation. Number inside each bar is the total number of oocytes evaluated. *<0.05 and ****<0.01.

GV oocytes continued to show low 2-cell embryo rates when compared to the control oocytes (P<0.0001, Fig. 6.5). The degeneration noted with control denuded oocytes that underwent IVF was higher than that of *in vivo* GV matured denuded oocytes but was not statistically significant (9.5% *vs*. 4.7%, P=0.05).

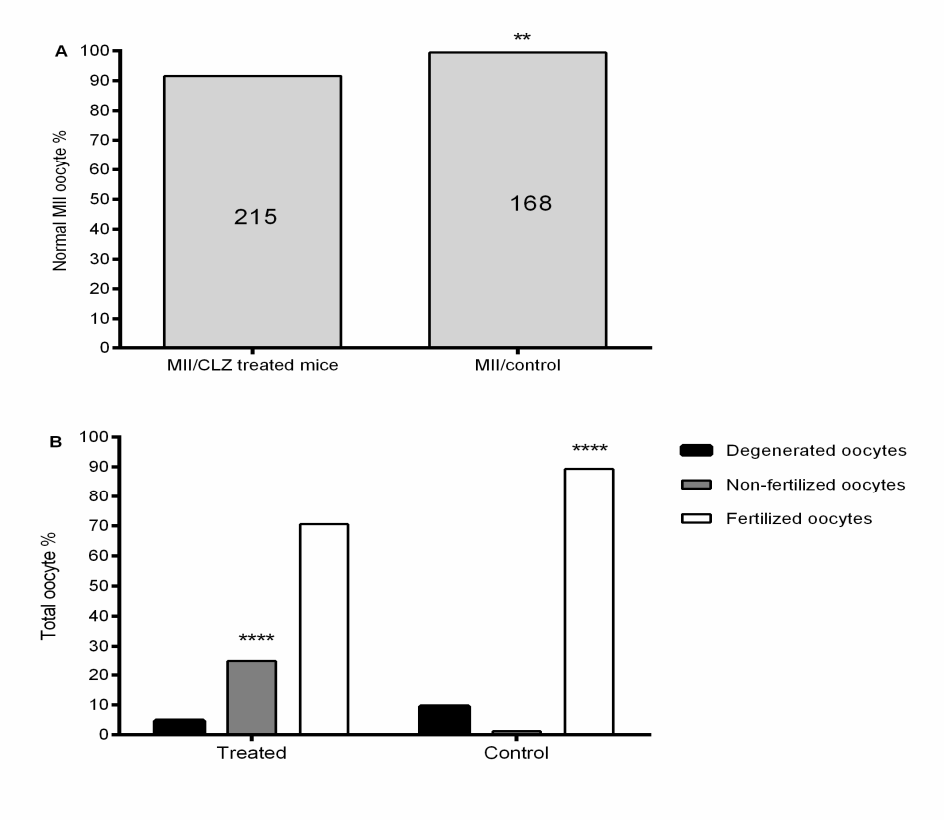

Figure 6.5. Normality and fertilization rates of GV oocytes *in vivo* matured in the oviduct. Mice were treated with 7.5mg CLZ at 4h pre-hCG and 2h post-hCG and oocytes were collected 24h post-hCG (IVOM). The oocytes were denuded and scored according to their normality with MII ovulated oocytes collected from control mice 14h post-hCG (A). The normally scored MII oocytes (denuded) obtained from IVOM were *in vitro* fertilized and compared to control MII oocytes that were also denuded (B).

Discussion

We observed here that ovulated MI oocytes from superovulated mice treated with CLZ can resume meiotic maturation in the oviduct or *in vitro* and that the resulting MII oocytes had maturation rates similar to those observed in untreated superovulated mice. Such oviductal or *in vitro* matured MI (into MII) oocytes have, upon IVF, high rates of 2-cell embryos when compared to those of ovulated MII oocytes from control mice. We then studied the IVF outcomes from ovulated GV oocytes and found that the GV oocytes were also capable of resuming meiotic maturation in the oviduct or *in vitro* and to yield MII oocytes at normal rates. Such MII oocytes exhibited higher IVF rates when the GV oocytes underwent IVM than that of control ovulated MII oocytes but the MII oocytes resulting from the oviductal maturation of GV oocytes had lower IVF rates. We observed that oviductal maturation of ovulated GV oocytes was associated with low rates of normal MII oocytes. Moreover, conducting IVF on only those normally scored MII oocytes continued to show lower rates of 2-cell embryos. We found that the administration of CLZ in superovulated mice was not only able to increase the IVF rate but also to synchronize meiotic maturation progression among oocytes and to synchronize maturational course time between IVM and IVOM of immature oocytes.

Scoring of ovulated GV oocytes matured *in vivo* or *in vitro* after 10h showed approximately 100% MII oocytes whereas GVBD was observed to occur around 1h *in vivo* or *in vitro* (Chapter III and IV). Conversely, scoring oocyte

maturation in superovulated mice not treated with CLZ showed emission of the 1st polar body as early as 6h post-hCG whereas occurrence of GVBD was first observed 3h post-hCG and continued to occur up to 9h post-hCG; the last time point tested in this mouse species (Chapter III). Also monitoring of IVM of ovarian GV oocytes showed MII maturation rate of 61.6% after 10h that continued to increase up to 14h in this mouse strain. These results show that temporal arrest of meiosis during superovulation is capable of synchronizing GVBD and 1st polar body emission in a uniform meiotic progression among oocytes in comparison to those observed in oocytes that have not been temporary arrested. This also directs attention to the occurrence of early polar body emission, and consequently ovulation of aged MII oocytes with compromised viability, quality, and fertility. This emphasizes the usefulness of synchronization of oocyte maturation in superovulation programs.

Furthermore, CLZ allowed for both *in vitro* and *in vivo* maturation of oocytes to be synchronized. Here, we observed similar *in vitro* and *in vivo* maturation periods of 3 or 6h for MI oocytes; depending on CLZ time of administration, and 10h for GV oocytes. Conversely, IVM of oocytes upon release from the follicle is about 14-18h in most mouse strains whereas *in vivo* maturation of oocytes in superovulated mice requires 12-13h; the time window between the ovulatory stimulus and ovulation. In fact, it is not only the timing of the 1st polar body emission that is different between *in vitro* and *in vivo* matured oocytes but also GVBD. While timing of GVBD in oocytes undergoing IVOM was

reported to be greatly varied from 1h to 8h post-hCG, depending on mouse strain, GVBD under IVM is found to be within 3h (Calarco *et al*. 1972; Chapters III and V; Edwards and Gates 1959; Hsieh *et al*. 2007; Kalous *et al*. 2006; Miao *et al*. 2004).

Administration of a PDE3-I in superovulated mice blocks meiotic maturation at the GV stage, allowing more oocytes to synthesis and to accumulate the necessary transcripts, proteins, and nutrients that aid in sustaining meiotic progression and foster early embryonic development (Krisher 2004; Watson 2007). This temporal arrest at the GV stage was also observed to allow oocytes to gain an advanced chromatin configuration of SN. Administration of gonadotropins in superovulated mice are known to increase SN configuration in oocytes but administration of CLZ in superovulated mice is believed to have a synergistic effect on having SN configuration in oocytes since intraoocyte cAMP was found to positively correlate with SN configurations in oocyte (Bouniol Baly *et al*. 1999; chapter V; de Lesegno *et al*. 2009; Nogueira *et al*., 2003a; Vanhoutte *et al*. 2007; Zuccotti *et al*. 1998 and 2002). In addition to SN configuration, superovulated mice treated with CLZ have oocytes with advanced cytoplasmic maturation of CG with intermittent domain (CGID), a pattern that precedes the formation of free domain in the peripheral CG pattern in MI oocytes. As a result, such oocytes that had more time to finish RNA synthesis, gain SN configuration, and display advanced CG distribution were found to yield higher rates of IVF than control ovulated MII oocytes.

Superovulation in mice was found to cause a delay in embryonic development, an increase in abnormal blastocyst formation, and pronounced fetal growth retardation (Van der Auwera and D`Hooghe 2001). Superovulation in hamster was reported to cause abnormal microfilament distribution and to adversely affect embryonic development (Lee *et al*. 2005). Moreover, a dose-response relationship between PMSG dose and the incidence of polyploidy was noted (Ma *et al*. 1997). In human superovulation, serial measurements of serum estrogen and applications of transvaginal ultrasonography are used to monitor the follicular development and to adjust hormonal dosage administrations (Tur *et al*. 2001). Nevertheless, a delay of embryonic development and low birth weights were also observed after IVF in humans (FIVNAT 1995; Sundstrom *et al*. 1997). It is suggested that the exogenous gonadotropins have a negative impact on oocyte maturation and consequently fertilization in addition to the observed effect of lack of synchronization of meiotic progression among oocytes. Administering CLZ in superovulation is believed to reduce some of these adverse effects since temporal arrest of meiosis is positively correlated with SN configuration, transcript and substrate accumulation, advanced CG distribution, and synchronization of meiotic events among oocytes (Chapter V).

Previous reports showed that *in vitro* arrest of oocytes at the GV stage using PDE-I was associated with higher oocyte quality, fertilization rate, and embryonic development when compared to those of non-arrested GV oocytes but lower than those of ovulated MII oocytes (Albuz *et al*. 2010; Nogueira *et al*.

2003a; Shu. *et al*. 2008; Vanhoutte *et al*. 2007 and 2008). This indicates the superiority of *in vivo* maturation and the beneficial effect of temporal arrest of meiotic maturation. On the other hand, other investigators have reported the lack of such beneficial effects on oocyte maturation and IVF rate (Curnow *et al*. 2011; Jee *et al*. 2009). It is possible that the concentration, the PDE3-I type, and/or the duration of arresting of oocytes at the GV stage are the factors that explain such discrepancies. While the *in vivo* arrest of oocytes at the GV stage followed by IVM improved the IVF rate more than that of ovulated MII or *in vitro* matured ovarian GV oocytes (Chapter V), the ovulated GV oocytes matured *in vivo* resulted in the lowest IVF rate because of the high degeneration rate and poor fertilization capability. Interestingly, oviductal maturation of MI oocytes for 3 or 6h in mice treated with a single CLZ dose showed no such degeneration or poor IVF rate. In humans, delaying IVF after oocyte collection resulted in high levels of fertilization, fetal development, and pregnancy (Patrat *et al*. 2012; Sundstrom and Nilsson 1988; Trounson *et al*. 1982). Moreover, delaying oocyte retrieval after the hCG injection showed similar results (Son *et al*. 2008). These results indicate that prolonged *in vivo*/vitro maturation of oocytes is beneficial and may increase cytoplasmic maturation. In our study, the incubation period for the oviductal maturation was similar to the duration of IVM, excluding the possibility of factor(s) undergoing aging in oocytes upon prolonged meiotic arrest and maturation. Therefore, it is suggested that other *in vivo* factor(s) such as CLZ

concentration upon multiple administrations may affect oocyte viability and normality.

CLZ has an elimination half life of 11-13h in humans, which is thought to be relatively similar to that of mice (Chapter III and IV). The compound also has active metabolites and high protein binding capacity of 95–98% (Lacy *et al*. 2011). Although delaying retrieval of GV oocytes in mice treated with 7.5mg CLZ, 4h pre-hCG and 2h post-hCG, resulted in mice ovulating mostly MI oocytes, only half of the mice treated with 7.5mg CLZ at the same time of hCG and 6h post-hCG yielded mostly MI oocytes upon delayed retrieval (Chapter III). Genetic polymorphisms in hepatic CYP 450; CYP3A (*CYP3A5*3*) and CYP2C19 (*CYP2C19*2* and *CYP2C19*3*), were found to cause substantial interindividual variability in CLZ clearance when administered orally (Yoo *et al*. 2010). Moreover, sex differences were also reported to affect pharmacokinetics of CLZ in rats (Kamada *et al*. 2011). It is possible that not all mice treated with multiple CLZ administrations could show similar CLZ metabolic profiles and consequently CLZ inhibitory concentrations could be maintained longer in some of the treated mice. As a result, the meiotic maturation kinetics may be disrupted. Another possibility for the poor oocyte fertilization potential of MII oocytes resulting from IVOM of ovulated GV oocytes is the observed ovulated GV oocytes with high rates of peripheral CGID whereas some of the ovulated MI oocytes had been reported to have exocytosed CG (Chapter V). The exocytosed CG status was reported to occur in fertilized, aged, or apoptotic oocytes whereas the CGID is a

transition feature between GV oocytes that mature to MI oocytes (Chapter V; Liu *et al*. 2003 and 2005). Moreover, it was reported that arresting oocytes at the GV stage by delaying the endogenous LH for one day in rats is associated with abnormal distribution of CG resulting in decreases in oocyte fertility and litter size (Stoker *et al*. 2003). It is possible that early activation or abnormality of CG is responsible of such low fertilization capacity. Those two factors; CLZ metabolic profile and premature CG activation, discussed here may explain the compromised fertilization rate in normal MII oocytes obtained from IVOM of GV oocytes but not the observed high rate of degeneration in the matured MII oocytes.

CLZ was reported to have cellular protective properties against reactive oxygen species and DNA fragmentation. The compound inhibits oxidative stress, augments metallothionein, and restores reduced glutathione levels (Lee *et al*. 2004 and 2010; Ota *et al*. 2008; Shin *et al*. 2004; Suzuki *et al*. 2007). Nevertheless, potential degenerative impact of CLZ or its metabolites could be the reason behind the notable degeneration in MII oocytes resulting from IVOM of GV oocytes. Prolonged maintenance of ovarian oocytes at the GV stage *in vitro* is harmful and leads to degeneration. Moreover, this degeneration increased as the CLZ concentration and incubation time increased. A sustained high intraoocyte cAMP level is suggested to be positively correlated with oocyte degeneration (Chapter II). The longer arrest of oocytes at the GV stage in the oviduct than that *in vitro* is likely if some of the CLZ treated mice had had

different CLZ pharmacokinetics resulted from poor metabolizing enzymes (as discussed in the previous section). It is believed that prolonged arrest of oocytes at the GV stage *in vivo* increases degeneration.

In addition to the synchronization of oocyte meiotic and cytoplasmic maturation and its positive consequences, it is observed in this study that synchronization of meiotic progression among oocytes *in vitro* or *in vivo* is possible. Moreover, both *in vitro* and *in vivo* maturation course times of immature oocytes were also synchronized. Consequently, ovulated GV or MI oocytes were fertilized and resulted in higher rates of 2-cell embryos than those of ovulated MII oocytes except for GV oocytes when they resumed meiosis *in vivo*. If CLZ is capable of showing the same meiotic inhibition in other species, such a technique may be adopted in IVF clinics to increase pregnancy rates. Nevertheless, further evaluation of this technique should address the entire oocyte competence including production of blastocysts, live births, and epigenetic effects.

Summary and conclusion

- Ovulated MI oocytes resulting from superovulated mice treated with 7.5mg CLZ, 7h pre-hCG, have similar maturation rates but higher IVF rates, upon *in vitro* or *in vivo* maturation, than ovulated MII oocytes resulting from superovulated mice not treated with CLZ.

- Ovulated MI oocytes resulting from superovulated mice treated with 7.5mg CLZ, 4h pre-hCG, have similar maturation rates but higher IVF rates, upon *in vitro* or *in vivo* maturation, than ovulated MII oocytes resulting from superovulated mice not treated with CLZ.
- Ovulated GV oocytes resulting from superovulated mice treated with 7.5mg CLZ administered at the same time as hCG and 6h post-hCG have similar maturation rates but higher IVF rates, upon *in vitro* maturation, than ovulated MII oocytes resulting from superovulated mice not treated with CLZ.
- Ovulated GV oocytes resulting from superovulated mice treated with 7.5mg CLZ administered at the same time as hCG and 6h post-hCG have similar maturation rates but with higher degeneration rates, upon *in vivo* maturation, than ovulated MII oocytes resulting from superovulated mice not treated with CLZ.
- Meiotic maturation of ovulated MI oocytes resulting from CLZ administered 4 or 7h pre-hCG or GV oocytes resulting from CLZ administered at the same time as hCG and 6h post-hCG can be synchronized to mature within the same time. Scoring the maturation state for those immature oocytes after 3, 6, or 10h, respectively, revealed that all oocytes matured into the MII stage.
- *In vitro* and *in vivo* meiotic maturation of ovulated MI oocytes, resulting from CLZ administered 4 or 7h pre-hCG, or GV oocytes, resulting from

CLZ administered at the same time as hCG and 6h post-hCG, can be synchronized to occur at a similar time. Scoring maturation status for those immature oocytes after 3, 6, or 10h, respectively, revealed that all oocytes matured into the MII stage whether oocytes were matured *in vitro* or *in vivo*.

- In addition to the high degeneration rate associated with MII oocytes resulting from *in vivo* maturation of ovulated GV oocytes, the non-degenerated MII oocytes also have poor fertilization capability.

CHAPTER VII

CONCLUSION

The contraceptive properties of CLZ were evaluated using mice. We found that CLZ is able to arrest spontaneous oocyte maturation *in vitro*, to inhibit oocyte maturation in superovulated mice with a wide range of times of administration, and to block pregnancy in naturally cycling mice.

Ovulated immature oocytes obtained from superovulated mice treated with CLZ were evaluated by comparing them with ovarian immature oocytes obtained from hyperstimulated mice and with ovulated mature oocytes obtained from superovulated mice not treated with CLZ. We concluded that the ovulated immature oocytes have an advanced cytoplasmic and meiotic maturation and higher fertilization rates (2-cell embryo) when compared to both ovarian immature and ovulated mature oocytes. We also concluded that synchronization of meiotic progression among oocytes or between *in vitro* and *in vivo* matured oocytes in superovulated mice is achievable when CLZ is administered.

During this study, we formulated the principle that CLZ is not only able to block oocyte meiotic maturation and pregnancy but also can be utilized to produce oocytes with high developmental levels and fertilization rates. From the contraceptive point of view, CLZ merits further evaluation as a potential non-steroidal contraceptive. From the higher developmental and fertilization point of

view, administration of CLZ in superovulation protocols merits continued investigation to address oocyte competence comprehensively .

Future studies are required to evaluate CLZ contraceptive effects in other animal models. Studies are also needed to address the different pharmacological responses of CLZ between mice and rats. Administration of CLZ in rats using the described method of administration in this study and/or administration of CLZ in mice using the manufacturer`s method of administration will aid to uncover the differences between mice and rats and the differences between the different methods of administration. Finally, comprehensive evaluation for the CLZ ability to improve oocyte quality and fertilization requires producing live offspring and evaluating their normality.

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