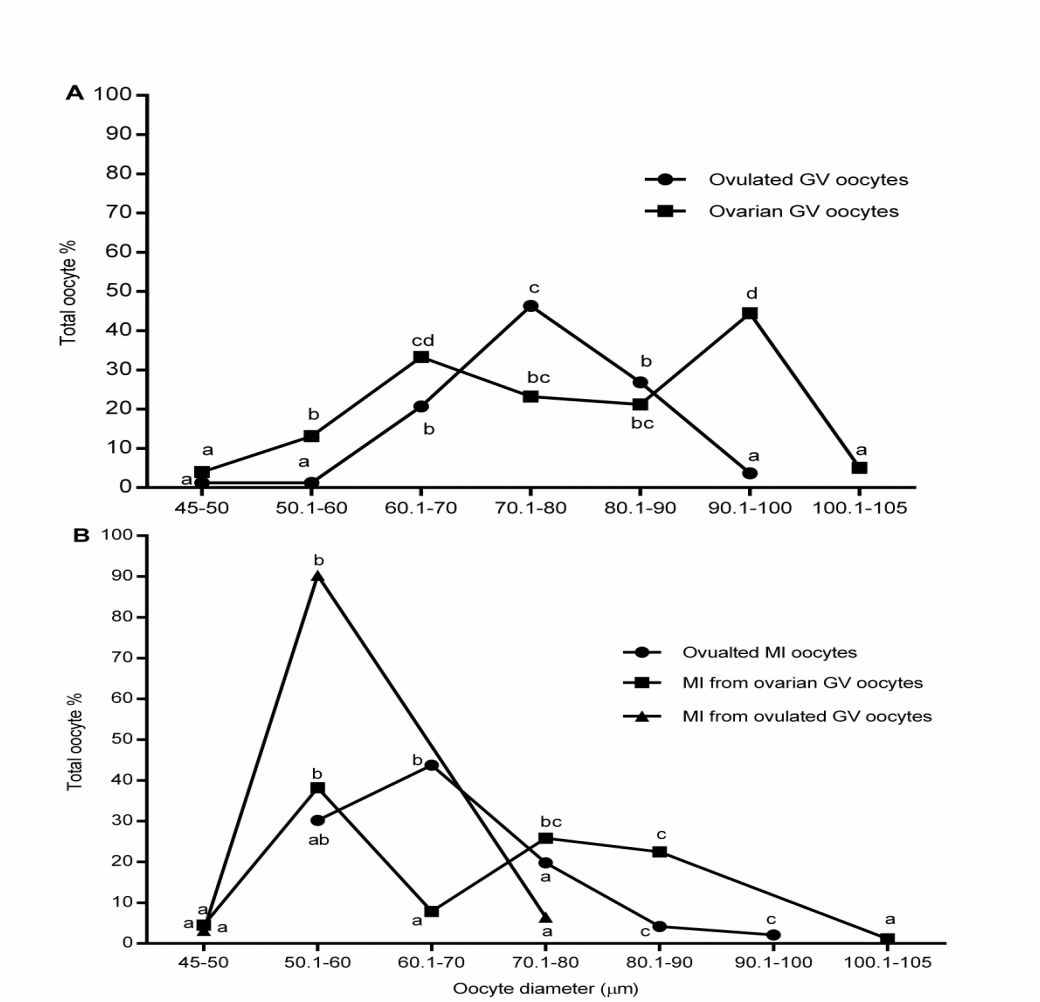

Figure 5.6. Diameter distribution of GV and MI oocytes obtained from different protocols. A: ovarian GV oocytes collected from antral follicles had a wider diameter size range of 50-100μm than ovulated GV oocytes that had a diameter size range of 60-90μm. B: note the uniform diameter size of ovulated 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).

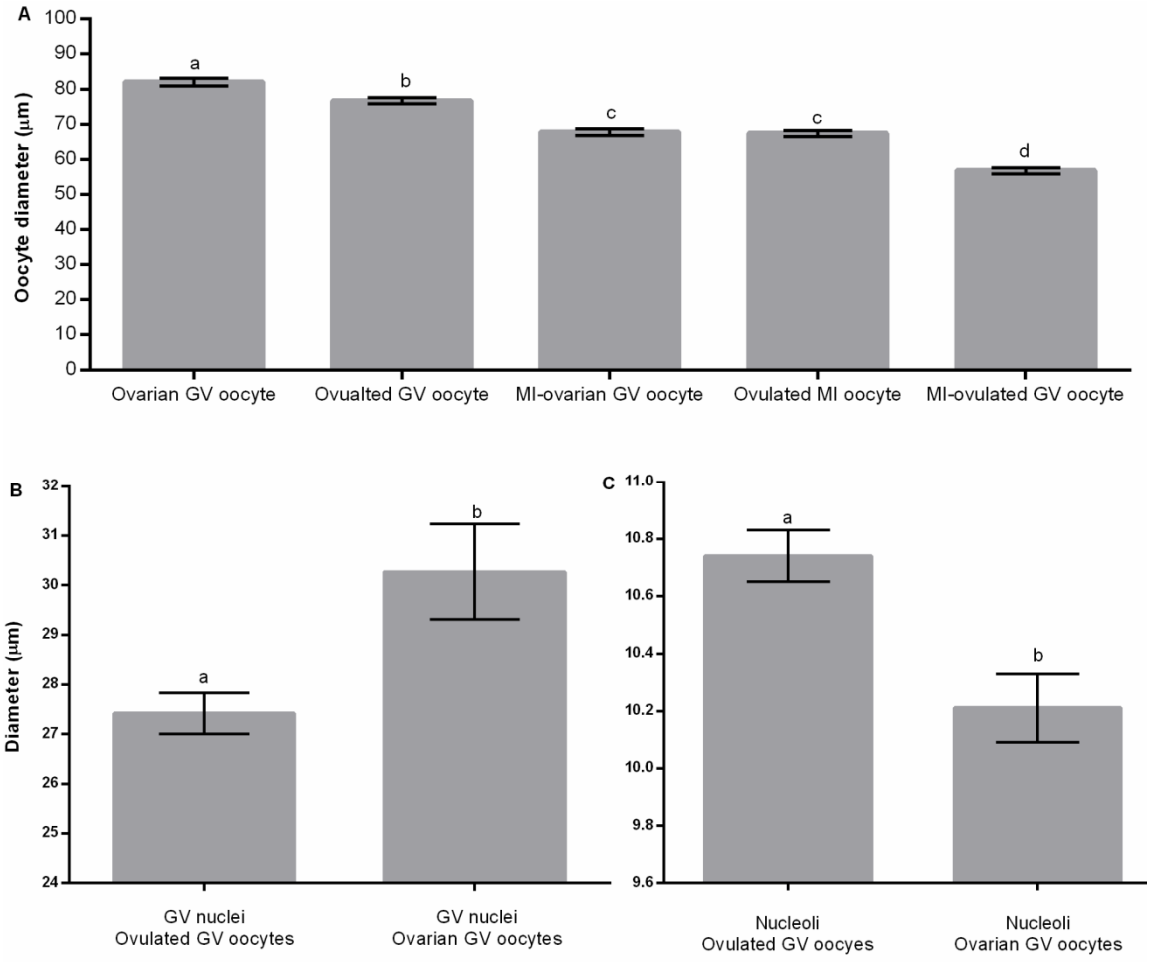

Figure 5.7. Oocyte, GV, and nucleolus diameters of different oocyte types. A: Diameters of oocytes of different origins and meiotic stages. Data points with different superscripts are significantly different (P<0.007). B: GV diameters of ovulated and ovarian GV oocytes (P=0.007). Nucleolus diameters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

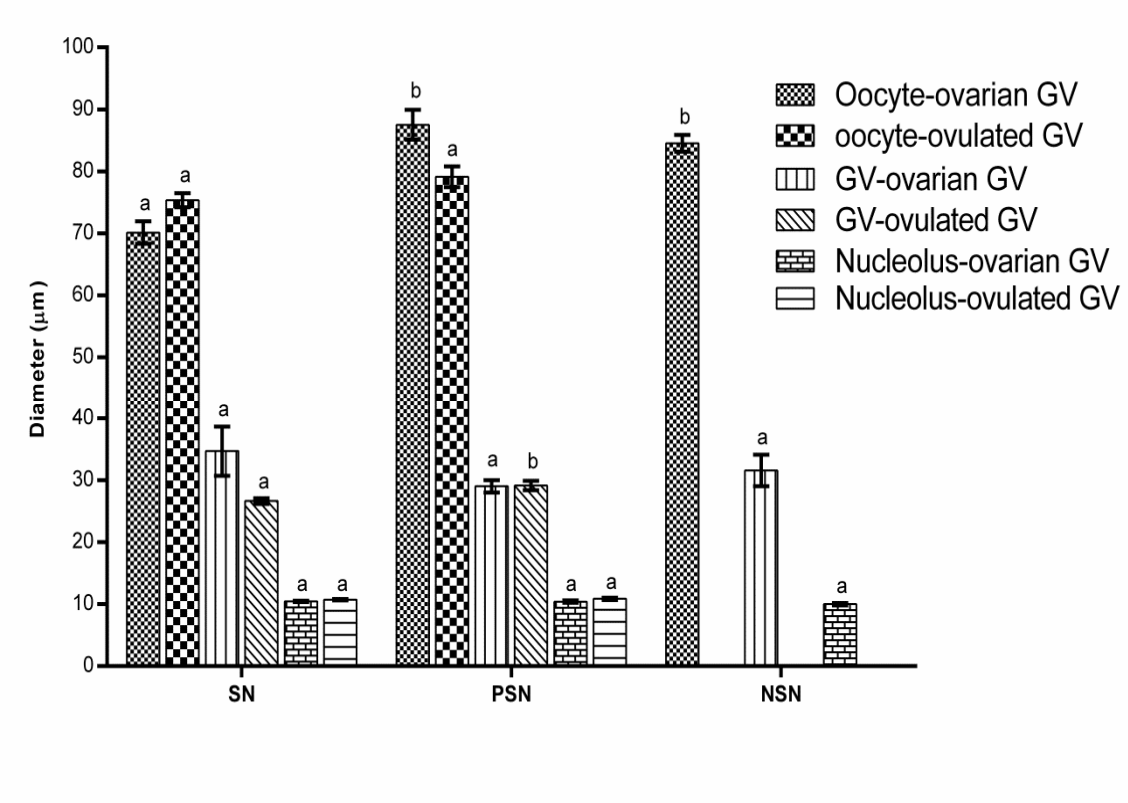

Figure 5.8. Oocyte, GV, and nucleolus sizes of ovulated and ovarian GV oocytes of different chromatin configurations. Different superscripts indicate significant differences among the different chromatin configurations. Data are presented as mean±SEM.

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.

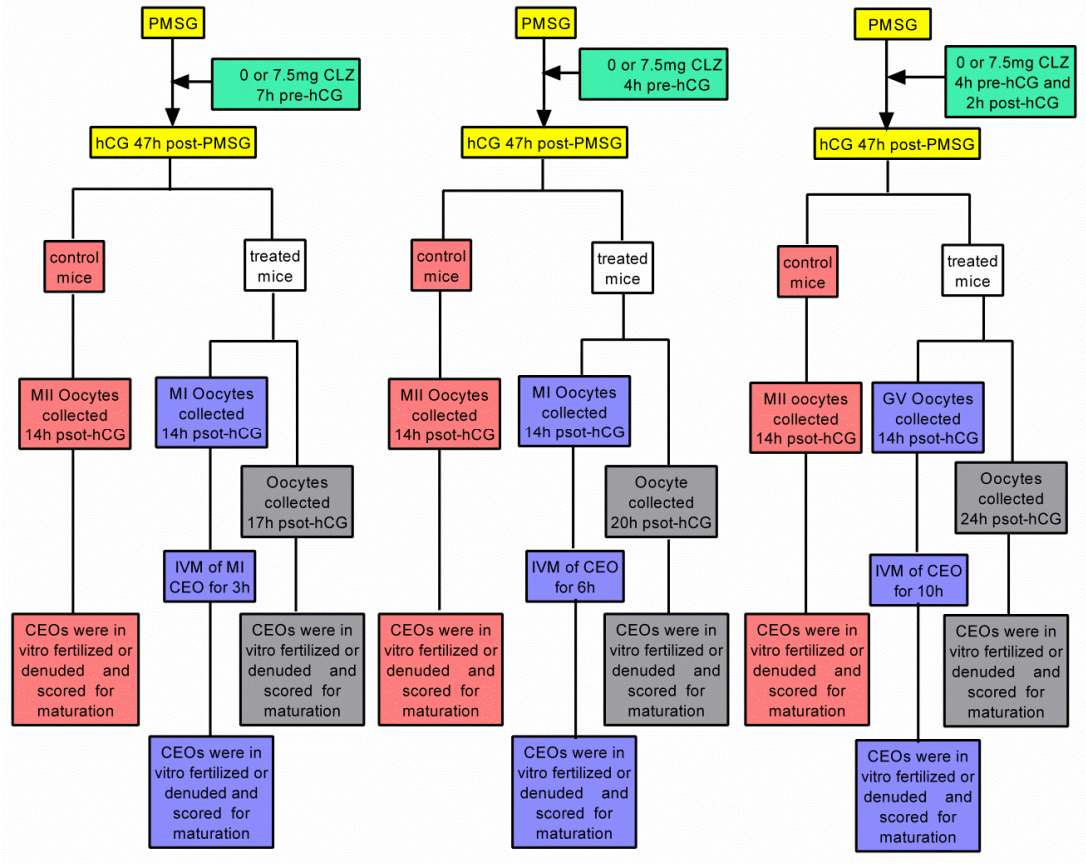

Figure 6.1. The experimental design of chapter six. Ovulated germinal vesicle (GV) and metaphase I (MI) oocytes were *in vitro* or *in vivo* matured (IVM or IVOM, respectively) to mature oocytes followed by *in vitro* fertilization (IVF) from superovulated mice; pregnant mare serum gonadotropin (PMSG) 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).

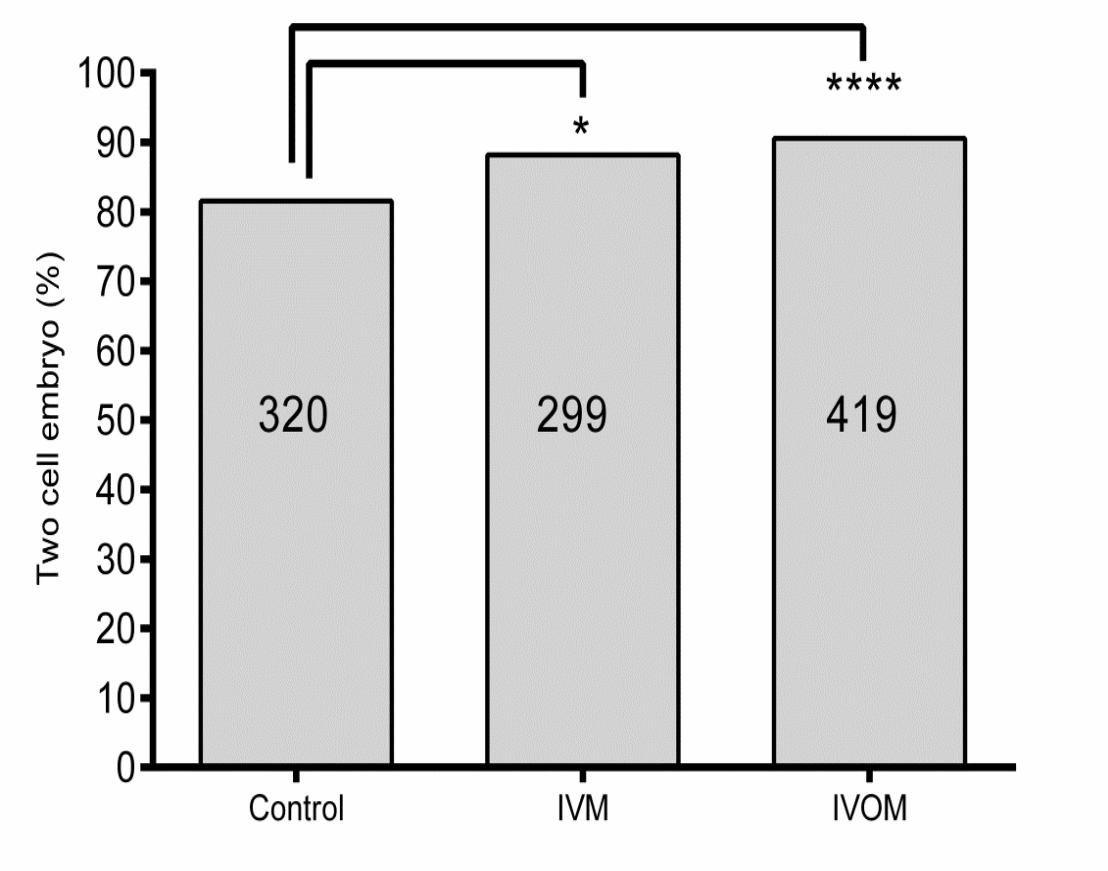

Figure 6.2. Two cell embryo rates for ovulated MI oocytes resulting from Mice treated with 7.5mg CLZ at 7h pre-hCG. Ovulated MI oocytes were collected 14h post-hCG followed by IVM for 3h and IVF or at 17h post-hCG (IVOM) followed by IVF. Oocytes from control mice were collected 14h post-hCG and underwent 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).

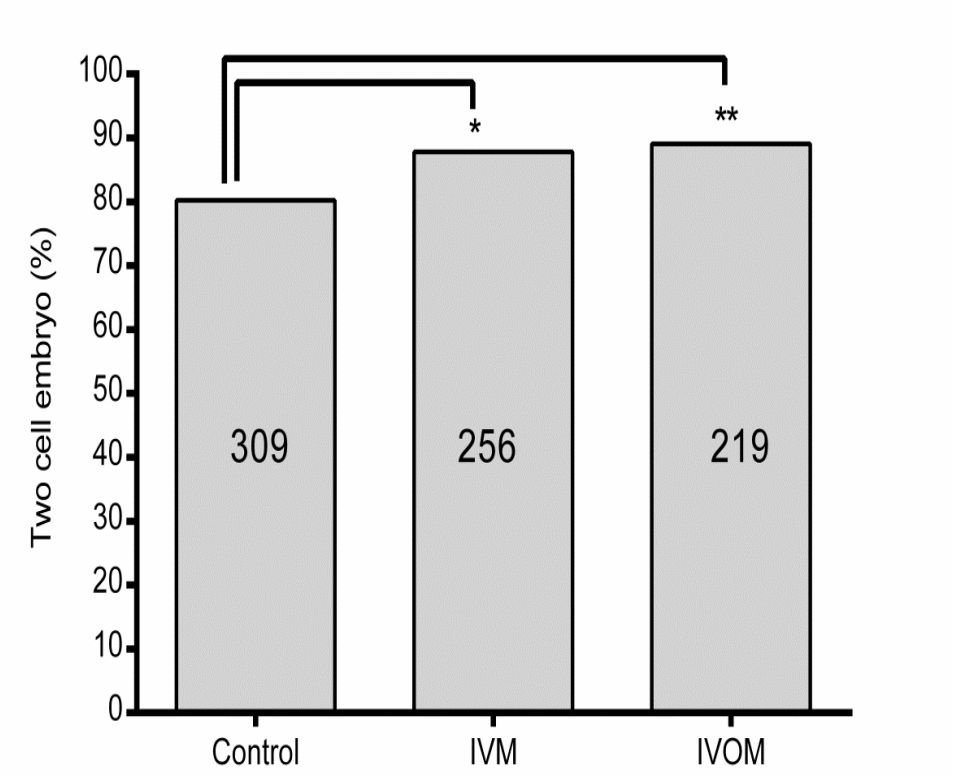

Figure 6.3. Two cell embryo rates for ovulated MI oocytes resulting from Mice treated with 7.5mg CLZ at 4h pre-hCG. Ovulated MI oocytes were collected 14h post-hCG followed by IVM for 6h and IVF or at 20h post-hCG (IVOM) followed by IVF. Oocytes from control mice were collected 14h post-hCG and underwent 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

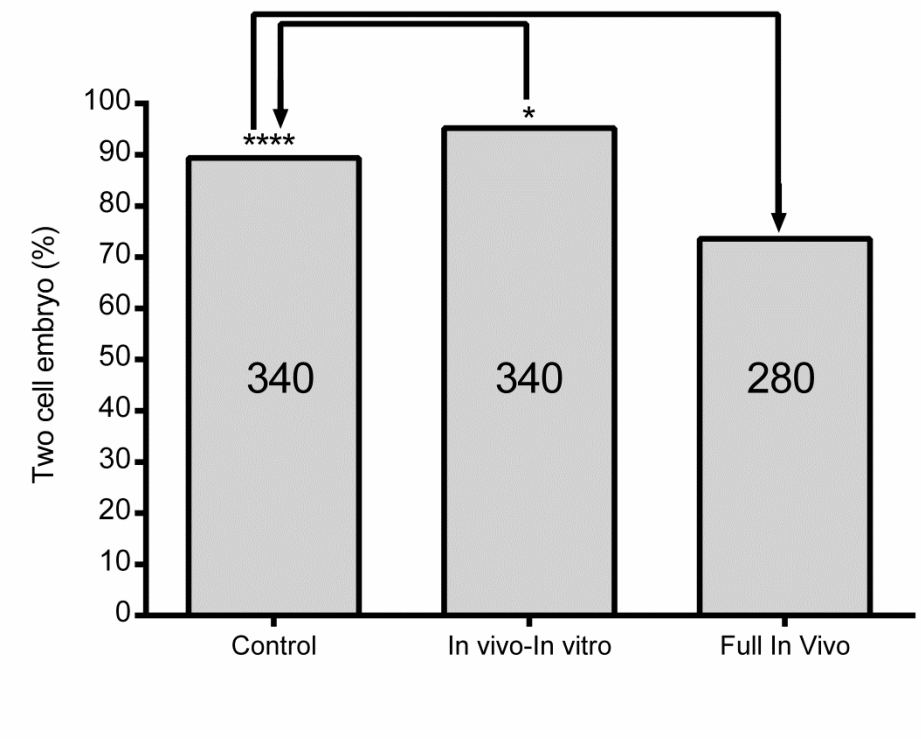

Figure 6.4. Two cell embryo rates for ovulated GV oocytes resulting from Mice treated with 7.5mg CLZ at 4h pre-hCG and 2h post-hCG. Ovulated GV oocytes were collected 14h post-hCG followed by IVM for 10h and IVF or at 24h post-hCG (IVOM) followed by IVF. Oocytes from control mice were collected 14h post-hCG 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).

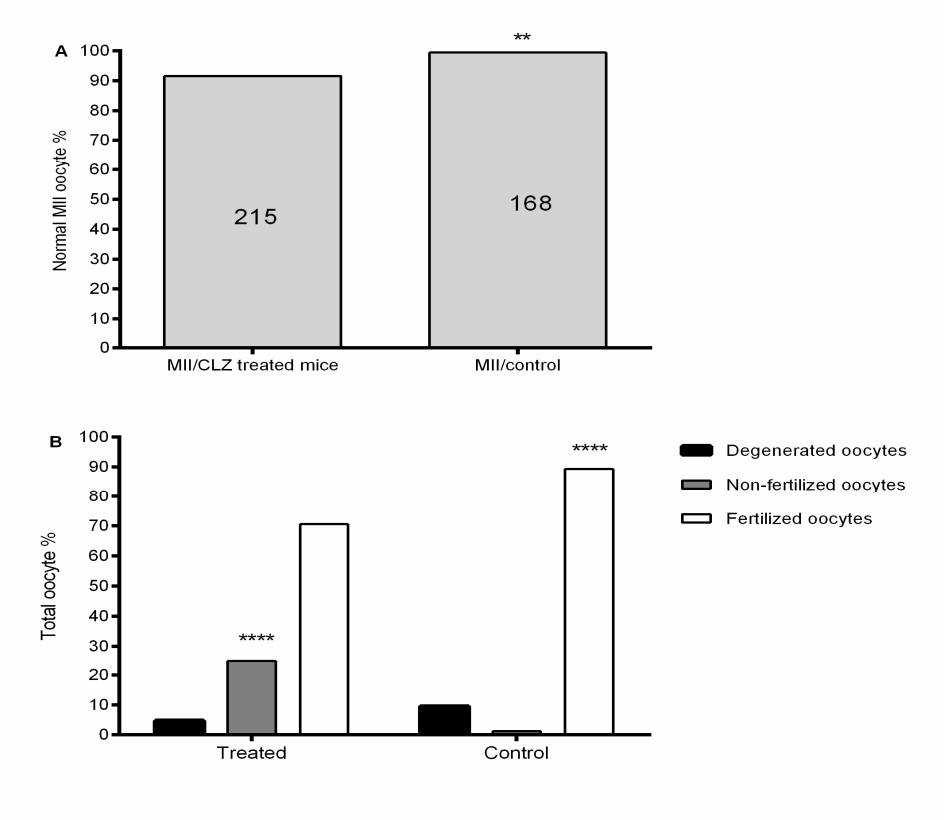

Figure 6.5. Normality and fertilization rates of GV oocytes *in vivo* matured in the oviduct. Mice were treated with 7.5mg CLZ at 4h pre-hCG and 2h post-hCG and oocytes were collected 24h post-hCG (IVOM). The oocytes were denuded and scored according to their normality with MII ovulated oocytes collected 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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