A Thesis for the Degree of Master of Science

Development Competence and Molecular Characterization of Quail Oocytes after Ovulation

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SUMMARY

Natural fertilization in avian species occurs in the infundibulum shortly after oocyte is ovulated. Once the ovum moves beyond the infundibulum, the secretions from magnum hinder sperm to interact with the oocyte, but intracytoplasmic sperm injection (ICSI) enabling the fertilization of oocytes from magnum has been demonstrated in past studies. However, in these studies, birds must have been sacrificed for oocyte collection. If the oocyte in the shell gland can be used for ICSI studies, animal ethnic issue of sacrificing birds can be avoided and it is expected that the study can be applied to save the endangered avian species. Accordingly, the objective of the first study was to determine fertilizing capability of the oocytes with the time elapsed after ovulation.

First, Japanese quail sperm and inositol 1,4,5-trisphosphate (IP_3) were injected by ICSI technique to induce *ex ovo* fertilization. Injection was performed to the oocytes collected at (1) 0.5-1.5 (2) 3.5-4 (3) 5-6 (4) 9-10 and (5) 22-23 hr after ovulation. The injected oocytes were cultured in DMEM inside a CO_2 incubator for 24 hr. In order to determine the fertilization and blastoderm development rates of the oocytes, the morphology of the blastoderms was observed under a stereo microscope and histological verification was conducted after staining with 4.6-diamidino-2-phenylindole (DAPI), respectively. The highest rate of fertilization (60%) was observed in oocytes collected at 0.5-1.5 hr post ovulation, whereas it ranged between 36.4-18.8% in oocytes collected at other hours. 16.7% of oocytes collected at 0.5-1.5 hr post ovulation were developed beyond Eyal-Giladi and Kochav (EG&K) stage IV. However, the development of the oocytes collected at later hours post ovulation ceased development before EG&K stage IV. In this study, the positive sign for fertilization was seen in oocytes collected at all hours post ovulation. This indicates that the

fertilization capability is maintained until the egg is oviposited. However, the embryonic development of the eggs collected from isthmus and beyond ceases before EG&K stage IV. Therefore, an additional treatment to reverse the aging process of the oocytes can assist the embryonic development.

In order to improve the fertilization rate and further embryonic development, it seems necessary to elucidate underlining mechanism of incompetency of oocyte development. Accordingly, the molecular changes in unfertilized oocytes were studied in relation to times after ovulation. Also, responsiveness of oocytes to IP_3 injection was evaluated in terms of changes in mRNA expression. Here, quantitative RT-PCR analysis was employed to assess expression of several mRNA transcripts in the oocytes which may be associated with decay of fertilization and development. In order to observe the expression of the selected genes, the unfertilized oocytes were collected at 0.5-1.5 hrs post ovulation for negative control. For the IP_3 injected group, the unfertilized oocytes collected at 0.5-1.5, 5-6 and 22-23 hrs post ovulation and injected with IP_3 using ICSI method. Both groups of oocytes were incubated in DMEM for 3 hrs and each of the oocytes was subjected to total RNA extraction and cDNA synthesis for the Real time PCR analysis.

ITPR3 mRNA expression was variable among the unfertilized and IP_3-induced groups. Hence, there was no significant difference. On the other hand, as for mRNA expression of *ITPR1* in the unfertilized oocyte and the IP_3-injected oocytes, the up-regulated expression was observed at 0.5-1.5 hr post ovulation but much lower expression was at 5-6 hr and 22-23 hr post ovulation. The same expression pattern in *ITPR2* mRNA was seen both in the unfertilized and IP_3-injected oocytes. These correlated expression patterns of up-regulation at 0.5-1.5 hr and low expression at later hours post ovulation may indicate that IP_3 receptors type 1 and type 2 are involved in the process of calcium release and oocyte activation. The same tendency of

gene expression patterns were observed for *BCL2L1*, *CDK1* and *MAD2L2*. The expression of *BCL2L1*, *CDK1*, *MAPK1* and *MAD2L2* was highly upregulated in the injected oocytes at 0.5-1.5 hr post ovulation when compared to oocytes at other time. This indicates that the oocytes 0.5-1.5 hr post ovulation showed the significantly high responsiveness to IP_3 compared to other oocytes. Therefore, the low mRNA expression of *ITPR1* and *ITPR2* may be associated with the low fertilization rate in oocytes collected from shell gland and oviposited eggs.

Since the fertilization competency is maintained until the egg is oviposited, if the reversal of the oocyte aging is possible, the oocytes collected from shell gland and oviposited eggs can be used for successful fertilization and embryonic development and finally hatch. The present study is the first step towards understanding the fertilization capability in the avian species and to apply the new findings to contribute towards saving birds from being sacrificed and protecting endangered avian species.

Keywords: Japanese quail, oocyte activation, fertilization, oocyte aging, ICSI

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LIST OF ABBREVIATION

BCL2L1: BCL2-like 1

BTG4: B-cell translocation gene 4

CaMKII: Calmodulin dependent protein kinase II

CASP3: Caspase 3, apoptosis-related cysteine peptidase

CDK1: Cyclin-dependent kinase 1

CSF: Cytostatic factor

CYCS: Cytochrome c

DAPI: 4,6-diamidino-2-phenylindole

DMEM: Dulbecco's modified eagle medium

ER: Endoplasmic reticulum

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GV: Germinal vesicle

ICSI: Intracytoplasmic sperm injection

IP_3: Inositol 1,4,5-triphosphate

ITPR1: Inositol 1,4,5-trisphosphate receptor, type 1

ITPR2:Inositol 1,4,5-trisphosphate receptor, type 2

ITPR3:Inositol 1,4,5-trisphosphate receptor, type 3

MAD2L2: Mitotic arrest deficient-like 2

MAPK1: Mitogen-activated protein kinase 1

ZAR1L: Zygote arrest 1-like

ZP: Zona pellucida

ZP2: Zona pellucida glycoprotein 2

ZPD: Zona pellucida glycoprotein D

CHAPTER 1

GENERAL INTRODUCTION

Despite the fact that avian species have become a valuable model system for various researches, mechanisms and crucial factors regulating avian reproduction, processes of fertilization and early embryonic development have not been clearly elucidated. Much of the information available from the past studies on oocyte activation, fertilization and early embryonic development are available from the mammalian species. However, there are many differences between these two species since avian species are oviparous and the embryonic development occurs *in ovo* outside the body. Also, birds are characterized with polyspermy while mouse and other mammalian species are monospermic. Uncovering the mechanisms of reproduction, fertilization and embryonic development for avian species could be advantageous in various research fields studying avian species as bioreactor and for producing transgenic chicken. Also, this information can be applied to conserve endangered avian species.

In birds, the female reproductive tract is called the oviduct. Avian oviduct is divided into several regions, including infundibulum, magnum, isthmus and shell gland (Chousalkar and Roberts, 2008). The natural site of fertilization for birds is the first part of the oviduct, the infundibulum. Once the ovulated ovum moves through infundibulum to enter magnum, secretions and the outer perivitellne layer cover the surface of the ovum making impossible for sperm to reach the oocyte (Bakst and Howarth, 1977). However, the past studies have shown that the oocyte collected from upper magnum is possible to be fertilized by intracytoplsmic sperm injection (Hrabia et al., 2003; Mizushima et al., 2010; Takagi et al., 2007). This technique allows for a direct injection sperm into the oocytes collected from the various parts of oviduct removing the physical barrier covering the oocyte.

In mammals, oocytes have an optimal window of time for the normal fertilization and embryonic development to occur. The optimal fertilization period for mouse and rat is less than 12 hrs and 14 hrs after the oocyte is ovulated, respectably (Miao et al., 2009). If fertilization does not occur within these optimal period, unfertilized oocytes undergo the process called "oocyte aging". Mammalian oocyte aging has been associated with low fertilization rate (Goud et al., 1999), abnormal embryonic development (Tarin et al., 1999) and apoptosis and cellular fragmentation (Miao et al., 2009). However, the optimal window of fertilization period and the mechanisms of oocyte aging process for avian species have not been established yet. Understanding these processes will be a crucial contribution towards studies on avian model system.

In chapter 2, we review the mechanism of mammalian oocyte activation and fertilization and factors that may be related to the regulation of fertilization and oocyte aging in avian species. In chapter 3, we determine the fertilization and developmental rate of Japanese quail oocytes collected at various hours post ovulation. In chapter 4, the expressions of the selected genes are studied in unfertilized oocytes and activated oocytes from various parts of the oviduct to determine whether these factors are involved in oocyte aging in birds.

CHAPTER 2

LITERATURE REVIEW

1. Fertilization and oocyte activation in mammalian species

1.1. Fertilization

The fertilization in most species involved a programmed sequence of molecular events result in the formation of a diploid zygote from sperm and egg (Tulsiani and Abou-Haila, 2011). The fusion between the egg and sperm is known to be mediated through a specific sperm protein, called fertilin, and integrin receptors on the surface of the egg membrane (Almeida et al., 1995). The fertilization includes processes of specific cell adhesion, cell signaling, exocytosis, cell migration, fusion and cell cycle regulation. The fertilizing sperm must bind to the extracellular matrix of the oocyte called zona pellucida. When the sperm binds to the zona pellucida, the head of the sperm releases a large, specialized secretary vesicle called the acrosome which penetrate through the zona pellucida of the oocyte (Yanagimachi, 1994). The past studies have shown that sperm bind to the zona pellucida by a carbohydrate mediated process (Wassarman et al., 2001). The zona pellucida layer is responsible for the species-specific binding of the sperm and egg. Once the zona pellucida is removed from the oocyte, the sperm can bind directly to the plasma membrane of the egg. This technique has been used in clinic for *in vitro* fertilization to evaluate the fertilizing ability of sperm by allowing the interaction between human sperm and hamster eggs with zona perllucida removed (Wramsby and Hansson, 1984). Therefore, the zona pellucida may act as sperm receptors that recognize the sperm from the same species (Wassarman et al., 2001).

It has been demonstrated that the zona pellcida of mouse egg is composed of three glycoproteins, ZP1, ZP2 and ZP3 (Wassarman, 1988). They are synthesized by growing oocytes. Specifically, ZP3 serves as a receptor for sperm in mouse. In mouse, egg-binding proteins (EBPs) are also present in the oocyte which complements the sperm receptor (Snell and White, 1996). Many candidate EBPs have been discovered through past studies such as zonadhesion (Gao and Garbers, 1998)and zona receptor kinase (Tsai and Silver, 1996).

A large secretory vesicle in the apical region of the sperm head is called acrosome. It is known that ZP3 is the natural inducer that trigger the acrosome reaction during the binding of sperm to the zona pellucida (Ward and Kopf, 1993). Stimulation of ZP3 by sperm activates G proteins by cross linking of GalTase. Then IP_3 receptors on acrosomal membrane are activated and the influx of Ca^{2+} ions induces acrosome reaction (Snell and White, 1996) . After the sperm is able to penetrate the zona pellucida by acrosome reaction, the sperm binds to the plasma membrane of the oocyte and fusion between the egg and sperm occurs. When this fusion occurs, the oocytes undergo a process referred to as oocyte activation (Miller et al., 2002).

Once the fertilization occurs the dormant sperm nucleus transformed into a functional nucleus called the male pronucleus. This male pronucleus formation involves the removal of the sperm nuclear envelope, decondensation of the chromatin, disassembly of the sperm nuclear lamina and reassembly of a nuclear envelope with pores and a lamina. The pronucleus also undergoes a swelling process (Collas and Poccia, 1998). In mammals, the first step of the sperm nuclear

transformation involves the reduction of the disulfide bonds of protamines. The disassembly of the sperm nuclear envelope and decondensation of the chromatin occurs during the metaphase II to telophase II transition of the oocyte. Then the sperm chromatin recondenses and decondenses again during the female pronucleus formation process (Adenot et al., 1991). These steps are required for adjusting the chromatin to a structure that is compatible with DNA replication, transcription and mitosis. In many of the mammalian species, these processes occur within 30 minutes.

Maternal factors are the maternal effect genes which accumulate in the oocyte during oogenesis which play a crucial role during the development of the embryo. Through many gene-knockout studies in the past, many mammalian maternal-effect genes have been identified. These include *Hsf1, Zar1, Npm2* and *Stella*. However, their molecular mechanisms have not been clearly understood. These maternal-effect genes should not affect oocyte maturation and ovulation but should impair the embryonic development (Ma et al., 2006).

HSF1, heat-shock factor-1 has been discovered to control the early post-fertilization development in mouse. The female mouse lacking HSF1 have normal ovaries and reproductive tract. Also, the egg is normally ovulated and able to arrest in metaphase II until fertilization and proceed to form two pronuclei post fertilization. However, the embryos die at 1-2 cell stage due to failure of zygotic transcriptional activity initiation. This activity is required for the maternal to embryonic control. Also the reduced survival may also be due disruption in structural integrity of the early embryonic nucleus (Christians et al., 2000).

Mater, a mouse oocyte protein dependent on the maternal genome,

is important for embryonic development beyond 2-cell stage. The first sign of embryonic transcription is detected in the late one-cell zygote stage in mouse. And this transcription is required for the development beyond the 2-cell stage. Exclusively expressed in oocytes, Mater transcripts accumulated during oogenesis. However, they were not detected in early embryos. Mater-null ova were fertilized normally in vivo and zygotes and 2-cell embryos showed the normal morphology. However, the development stopped at the 2-cell stage or showed degeneration. Therefore, in Mater- null females, zygotes can development normally through the first cleavage. However, the development is arrested at the 2-cell stage (Tong et al., 2000).

Matured oocyte and the one-cell embryo are transcriptionally silent but translationally active. The maternal to zygotic transition occurs during the late one-cell and early two –cell stages (Ma et al., 2006). In mouse, the preimplantation development can be divided into two phases. The first phase is the oocyte-to-embryo transition which is one to 2-cell stage. And the second phase is the 4-cell stage when the cellular differentiation occurs (Wang et al., 2004). It has been proposed that follicular fluid from growing follicles contains steroids and growth factors. Human oocytes contain mRNA for the estrogen receptor and receptors for insulin-like growth factor (IGF) and epidermal growth factors (EGF) and their receptor. Also, activin and inhibin secreted from granulose cells have been known as intrafollicular growth factors. These factors have been reported to promote the in vitro maturation and fertilization of primate oocytes (Alak et al., 1996).

cAMP, which is produced from granulose cells and transported to the oocyte via gap junctions, is important in maintaining the meiotic arrest

of the oocyte. In Xenopus oocytes, the phosphorylation of the p34cdc2 subunit of MPF was done by the high comcentration of cAMP-dependent protein kinase A. This results in an inhibition of MPF (Rime et al., 1992).
M-phase promoting factor or maturation promoting factor (MPF) was first characterized in 1971 in mature *Xenopus* oocytes (Masui and Markert, 1971). Many past studies have shown that MPF is composed of cyclin B, the regulatory subunit, and $p34^{cdc2}$ (Gautier et al., 1990; Pines and Hunter, 1989). In mammalian oocyte, two peaks of high MPF activity can be detected. The first peak is at the time of meiotic division resumption and the second peak during the metaphase II arrest. The high level of MPF is sustained for the second peak (Mattioli et al., 1991). It has been found that the high level of MPF between meiosis I and II inhibits DNA replication, reducing the cell division (Fulka et al., 1992). It has been proposed that RNA polymerase may be inhibited by MPF (Cisek and Corden, 1989). A protein kinase c-mos is proposed to increase the MPF activity in oocytes between the meiosis resumption and fertilization (Sagata et al., 1989). Once the fertilization occurs, the c-mos mRNA level rapidly decrease in mouse ooycte (Goldman et al., 1988).

The oocyte to embryo transition of the mammalian species lasts for a long time. Specifically in mouse, the developmental transition from oocyte maturation to embryonic genome activation takes over 40 hours (Howlett and Bolton, 1985). It has been shown that nuclear reprogramming must be completed by the time the embryonic genome is completely activated, which is at the end of the second cell cycle, for the successful production of viable embryos. From the oocyte maturation to the transcriptionally silent stages of the embryonic development, three different mechanisms act to change the ooplasm. These include the translation of stored maternal transcripts providing the cytoplasm with

new proteins, the post-translational modification of the existing and newly synthesized proteins and the degradation of proteins and mRNAs that are no longer needed. (Evsikov and Marin de Evsikova, 2009). Many of the maternal mRNAs are degraded during the early steps of the oocyte to embryo transition and these mRNAs may play an important role during oocyte growth. For example, mRNAs for zona pellucida are abundant in fully-grown oocytes but are not translated. Then are no longer detectable in ovulated mature oocytes (Evsikov et al., 2006).

Recent studies have shown that maternal microRNAs (miRNAs) also play essential roles during oocyte to embryo transition in both zebrafish and in mouse (Giraldez et al., 2006; Tang et al., 2007). Also, it has been reported that small interfering RNAs (siRNAs) may also important in gene expression in oocytes (Watanabe et al., 2008). Important proteins involved in processing of miRNA and siRNA are DICER 1 and AGO2 (Tam et al., 2008; Watanabe et al., 2008). Female sterility with abnormal oogenesis is resulted when *Dicer 1* was lost in the growing oocyte (Tang et al., 2007)

1.2. Oocyte activation

When the fertilizing sperm penetrates the zona pellucid by acrosome reaction and binds to the oocyte plasma membrane, the fusion between sperm and egg occurs. This induces a cascade of downstream signaling which are responsible for the process known as oocyte activation. In all species, the oocyte activation is characterized by the rise in Ca^{2+} concentration in the cytoplasm of the oocyte by the fertilizing spermatozoa

(Stricker, 1999).

In mammals, a series of Ca^{2+} oscillations are required for the normal embryonic development (Miyazaki et al., 1993). The term "Ca^{2+} oscillation was first described in 1990 to refer to the repetitive elevation of Ca^{2+} concentration in cells (Berridge, 1990). Also the term "Ca^{2+} spike" involves a Ca^{2+} transient with a sharp threshold with the amplitude independent from the intensity of the stimulus (Meyer and Stryer, 1991). During the fusion of sperm and egg, the fertilizing sperm releases sperm factor, known as phospholipase C zeta (PLC zeta) into the cytoplasm of the egg (Saunders et al., 2002). As a oocyte-activating factor, PLC zeta produces 1,4,5-inositol triphosphate (IP_3) and diacylglycerol (DAG) by cleaving phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3, then, binds to its receptors on the membrane of the endoplasmic reticulum (ER), which is the main store of Ca^{2+} in cell. This triggers the opening of the Ca^{2+} channels on the ER and Ca^{2+} ions are released into the cytoplasm of the oocyte (Lee et al., 2006b).

Two different hypotheses for the calcium transients during fertilization have been proposed: the receptor-mediated hypotheses and the sperm-factor hypotheses. In the receptor-mediated hypothesis, the sperm itself triggers the calcium rise by binding to the receptors located on the oocyte surface (Foltz and Shilling, 1993). On the other hand, the sperm-factor hypothesis proposed that upon gamete fusion, the sperm releases internally acting molecules into the cytoplasm of the oocyte to trigger the calcium rise (Swann, 1990). In mammalian species, the latent period of the fertilization supports the sperm-factor hypothesis. This was supported by the study of intracellular injection of sperm extracts into the oocyte which generated a calcium response (Sakurai et al., 1999; Stricker, 1997).

Further studies have shown that the intracellular injection of sperm extracts in to the oocyte was able to activate the oocyte and form polar body and even cleavage (Wu et al., 1997). Using intracytoplasmic sperm injection of the sperm extract into the oocyte produced not only calcium oscillation but also healthy offspring (Palermo et al., 1996). The results of these studies showed that the interaction between the sperm and receptors on oocyte surface is not required for the normal fertilization.

It has been shown in the past studies that the calcium transient is species-specific at fertilization with the differences in the frequencies of the calcium oscillation (Jones et al., 1998). Many studies have observed that a single calcium pulse was not enough to fully activate the oocyte but multiple calcium transients were required for the successful release oocyte from the meiotic arrest (Ozil, 1998; Stricker, 1996). In mouse, the first Ca^{2+} rise occurs approximately 1-3 minutes after the fusion of sperm and egg occur (Lawrence et al., 1997). The following Ca^{2+} transient is in a form of a wave that starts at the sperm entry site and the Ca^{2+} transient propagates across the oocyte (Nakano et al., 1997). It has been measured that the first Ca^{2+} transient lasts for about 3-4 minutes then the following Ca^{2+} transients lasts for less than 1 minute (Miyazaki et al., 1993; Swann, 1992). Ca^{2+} oscillations in mouse oocyte continue for several hours until the oocyte enters the interphase, the time of the male and female pronuclei formation (Jones et al., 1995)

In mammalian species, three different genes of IP_3 receptors are identified (Patterson et al., 2004). All three isoforms of IP_3 receptors are expressed in somatic cells. However, in oocytes, IP_3 receptor-type 1 (IP_3R1) is predominantly expressed (Fissore et al., 1999). IP_3R1 is consisted of three domains: an IP_3 binding domain, a regulatory domain

and a Ca^{2+} channel domain. The regulatory domain has multiple phosphorylation sites targeted by calmodulin/Ca^{2+} regulated kinase (CaMKII), protein kinase C (PKC) and M-phase kinases (MAPK) (Patterson et al., 2004). The binding of IP_3 to its receptors initiates the Ca^{2+} oscillation in mammalian oocyte. The oscillation ceases after a few hour at the time of pronuclei formation (Lee et al., 2006b).

Ca^{2+} oscillation is responsible for the activation of the oocyte and embryonic development by triggering processes of resumption of meiosis, block to polyspermy, recruitment of maternal mRNA and the activation of embryonic genome (Ducibella et al., 2006). Also, the embryonic development is influenced by the number and amplitude of Ca^{2+} transient during oocyte activation (Ozil et al., 2006). Oocyte obtains the ability to generate Ca^{2+} oscillation during oocyte maturation, from the prophase of the first meiotic division to the metaphase II arrest of the second meiosis. The mature mouse oocyte showed longer lasting Ca^{2+} oscillation with higher amplitude than the immature oocyte (Carroll et al., 1994).

The oocyte of the mammalian species is arrested at the diplotene stage of the meiosis I. This stage is also known as germinal vesicle (GV) stage. During each estrus cycle, the LH surge stimulates the oocytes to be released from the arrest and continue with resumption of meiosis which is induced by germinal vesicle breakdown (GVBD) (Fan and Sun, 2004). The oocyte completes the first meiosis and enters the second meiosis. The oocyte is arrested at metaphase II (MII) until it is fertilization. The oocyte arrest in metaphase II is regulated by maturation (or M-phase) promoting factor (MPF) and cytostatic factor (CSF) (Tunquist and Maller, 2003). In mammalian oocytes, cyclin B1 must be continually synthesized to maintain the high level of MPF which keeps the oocyte in metaphase II

arrest (Jones, 2004).

Maturation promoting factor (MPF) is composed of the regulatory unit called cyclin B and the CDK1 subunit (Gautier et al., 1990). Once the fertilization occurs, the oocyte is released from the arrest triggered by the decreased activity in MPF level due to degradation of cyclin B by anaphase promoting factor (APC) (Clute and Pines, 1999). The fertilizing spermatozoon enters the oocyte to release sperm factor, which trigger the release of Ca^{2+} from ER. Free Ca^{2+} ions in the cytoplasm bind to the calmodulin to activate CaMKII (Markoulaki et al., 2003). This causes emi degradation which in turn activates anaphase promoting factor (APC). The activated APC ubiquitinates cyclin B of MPF. This lowers the level of MPF and the oocyte is released from the mephase II arrest and meiosis resumption occurs (Tripathi et al., 2010).

2. Fertilization and oocyte activation in avian species

2.1 Fertilization

It has been reported in chicken that the ovulation occurs about 24 minutes after the previous oviposition (Morris, 1973). The formation of the first cleavage furrow occurs 4.8-6.7 hours post previous oviposition. According to studies with microscope, about one hour after ovulation, the spermatozoa entered the cytoplasm of the oocyte and transformed into pronuclei (Waddington et al., 1998).

In birds, the female reproductive tract is called oviduct and it can be distinguished into several different segments. Once the oocyte is ovulated, it moves along this tract for about 23 to 24 hours before it is expelled from the body, which is termed oviposition. The first part of the oviduct is called infundibulum and the opening is shaped like a funnel which actively engulfs the mature, ovulated ovum. The egg spends up to 20 minutes inside the infundibulum and moves into the second part of the oviduct, called magnum. This is the place where the egg-white proteins, thick and thin albumens are secreted and deposited on the ovum as it travels down this region for about 3 hours. The next segment is called isthmus, the site of outer and inner shell membrane formation. Ovum spends approximately 18-19 hours in shell gland, where the ovum absorbs water and $CaCO_3$ (Calcium carbonate) is deposited to form the components of the egg shell(Mongin and Carter, 1977). When the egg reaches the end of shell gland, it is expelled by the smooth muscle contraction and the egg is oviposited through the vagina to the outside of the female bird's body. It has been known that the natural fertilization in avian species occurs only in the infundibulum. When the ovum reaches the magnum, it obtains thick secretions which hinder sperm to interact with ovum (Bakst and Howarth, 1977).

During the fertilization of the avian oocyte, the spermatozoa is released from the storage tubules in the infundibulum (Bakst and Howarth, 1977). Then they bind to the inner vitelline membrane to undergo an acrosomal reaction. This process involves making holes in the membrane by releasing hydrolytic enzymes (Okamura and Nishiyama, 1978). In avian species, multiple sperm penetrate the inner perivitelline layer of the oocyte to enter the germinal disc (Perry, 1987). Perivitelline layer covers

the ovum of the chicken as an analogous to the zona pellucida (ZP) of the mammalian oocyte (Bakst and Howarth, 1977). This outer membrane of the mammalian oocyte acts as a sperm receptor. However, in avian species, another factor assists the sperm to penetrate the perivitelline membrane and enter the oocyte. Sperm-associated body is a hole found in the inner vitelline membrane found in Japanese quail. It was reported that there is no preferential localization sperm-associated bodies in the vitelline membrane around the egg. The sperm binds to the sperm-associated bodies by the posterior end of sperm flagella and help the sperm to penetrate the inner vitelline membrane (Rabbani et al., 2006). It has been reported that this sperm-associated bodies are produced by secretory cells in the luminal epithelium and aggregate to 20-80 μm in diameter. It has been suggested that the sperm-associated body materials are provided to ovulating eggs and scattered in the vitelline membrane layer of the oocyte (Rabbani et al., 2007).

In the secretory canals of the gland at the infundibulum, the heads of spermatozoa were found. However, the flagella were observed around the sperm-associated bodies on the luminal epithelium of quail (Sultana et al., 2004). Several studies showed that the flagellum of the sperm is bound to the posterior side of the sperm-associated body (Rabbani et al., 2007; Woolley and Bozkurt, 1995).

As in mammals, the fertilization in avian species also consisted with multiple sequential steps. First, the sperm interacts with the surface of the egg. In mammals, the oocytes are covered in envelope called zona pellucida (ZP) (Howes and Jones, 2002). The binding of sperm to ZP triggers exocytosis of the acrosomal substances from the sperm head such as proteases and glycosidases. They are essential to lyse the egg envelope.

In mice, three kinds of ZP glycoproteins (ZPA/ZP2, ZPB/ZP1 and ZPC/ZP3) were identified. In chicken, once spermatozoa are bound to the inner vitelline membrane, they undergo acrosomal reaction induced by ZPC or ZP1 (Takeuchi et al., 1999). There are six glycoproteins (ZP1/ZPB1, ZPA/ZP2, ZPB/ZP4, ZPC/ZP3, ZPD and ZPAX) have been identified for chicken (Goudet et al., 2008).

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2.2 Blastodermal development

The nuclear events and early embryonic development of the avian species have been studied since Harper published his study on the pigeon (Harper, 1904). Since, then many researchers have been done on domestic fowl (Bakst and Howarth, 1977) to show how the spermatozoa penetrated the ovum. Also, the first cleavage division of avian ovum was described by Bekhtina (Bekhtina, 1960). During the first hour after the preceding oviposition, the ovum is surrounded by little or no albumen and located in the infundibulum or anterior magnum. Then between 1.2 the 1.6 hour after the previous oviposition, the ovum is located in the anterior region of the magnum. At this time, the female nuclei were observed to be in metaphase (Perry, 1987). At this time, the spermatozoa were observed in the cytoplasm of the ovum. Once the ovum moves into the mid to posterior region of the magnum, it is surrounded by a thick layer of albumen. The pronuclei observed in the germinal discs enlarge in diameter from 7 to 17 µm. During the 3.5 to 4.5 hours post previous oviposition, the ovum can be found in the isthmus and the shell membrane is partially or completely formed (Perry, 1987)

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The embryonic developmental stages prior to oviposition are called Eyal-Giladi and Kochav (EG&K) stage. EG&K stages are divided into two larger periods. The first period is called the cleavage period. It consists of Stages I – VI. The cleavage period is marked with the cleaving of the germinal disk. Stage I oocyte can be collected from the hens about 5.5 to 6.5 hour after the previous oviposition. The germinal disc of this stage shows cleavage furrow at the central region. By stage III, about 80-90 blastomeres and at stage IV, around 300 cells are observed. As the stage of the blastoderm progresses, the beadlike appearance is lost. The period between stages VII and X is characterized with the formation of area pellucida (Eyal-Giladi and Kochav, 1976).

3. Molecular changes in oocyte aging

It has been known that the developmental potential after fertilization is depended on the oocyte quality (Wang and Sun, 2007). Generally oocyte is fertilized soon after it is ovulated, during a period of window for optimal fertilization. This window of period for optimal fertilization differs for each species (Miao et al., 2009). If the oocyte is not fertilized within this time period, it undergoes the process known as oocyte aging (Takahashi et al., 2000). Past studies on mammalian oocytes have reported that oocyte aging is associated with decreased fertilization rate (Goud et al., 1999) and abnormal embryonic development (Sakai and Endo, 1988). Also, premature decrease in the level of MPF and MAPK can be observed in aged oocytes (Liang et al., 2007; Tatone et al., 2006; Tian et al., 2002). Also, abnormal calcium oscillation can be observed in aged oocytes which

could induce apoptosis and cellular fragmentation (Gordo et al., 2002). The cellular fragmentation and apoptosis in aged oocytes can be also triggered by the disruption in mitochondrial membrane potential. Mitochondria can no longer regulate the release of pro-apoptotic and anti-apoptotic factors (Wilding et al., 2001). The mRNA level of the anti-apoptotic regulator, BCL2, is reported to be reduced in aged mouse oocyte (Gordo et al., 2002).

3.1 Abnormal Ca^{2+} elevation

In mammals, fertilization triggers a prolonged series of intracellular Ca^{2+} oscillation by IP_3. It has been reported that in aged mouse oocyte, the fertilization triggers a significantly higher frequency of Ca^{2+} elevation that in fresh oocytes although the amplitude of the oscillations in aged oocytes is smaller than that of fresh oocytes (Igarashi et al., 1997). The significant increased duration of Ca^{2+} elevation may result in disruption in Ca^{2+} regulation (Takahashi et al., 2000). This abnormal Ca^{2+} elevation results in a disruption in Ca^{2+} homeostasis which induces apoptosis and cellular fragmentation. This is associated with the breaking the equilibrium between anti- and pro-apoptotic factors during postovulatory oocyte aging which turns the triggers the apoptosis by the Ca^{2+} signaling (Gordo et al., 2002). It has been also reported that caspase 3 truncates IP_3 receptors which cause the leakage in Ca^{2+} ions. However, aged oocyte can no longer compensate for the uptake of the excess Ca^{2+} ions inducing aopotosis (Verbert et al., 2008).

3.2 Apoptosis and fragmentation

Some studies have shown that dysfunction of mitochondria and endoplasmic reticulum of the aged oocytes in mammalian species. This causes the mitochondria to release apoptogenic factors inducing cellular fragmentation (Wang, 2001). This in turn triggers the release of cytochrome c from the mitochondria and induces the cascade of caspase activity. The caspase activity is known to be regulated by the pro-apoptotic and anti-apoptotic Bcl-2 family of proteins. However, Bcl-2, the anti-apoptotic gene, is observed to be down-regulated in aged oocytes and negatively affects the function of Ca^{2+} pimp which trigger the abnormal Ca^{2+} elevation (Ma et al., 2005; Tatone et al., 2006).

3.3 Reversal of oocyte aging

The ways to reverse the oocyte aging have been actively studied in mammalian species. Kikuchi was the first to suggest the reversal of oocyte aging by regulating the level of MPF (Kikuchi et al., 2000). Since then other studies have reported that MAPK is also important along with MPF for the reversal process (Liang et al., 2007; Tian et al., 2002). During oocyte aging in mammals, the activities of MPF and MAPK occur earlier and faster in aged oocytes then in fresh oocytes. It was reported the aged porcine oocytes contained inactivated MPF by phosphorylation, also termed pre-MPF. This maybe be associated with the decreased MPF activity in porcine aged oocytes by misbalancing the kinase and phosphatase activities (Kikuchi et al., 2002). It was suggested that controlling MPF activity may be able to delay the oocyte aging process *in vitro*. The controlling of MPF was shown to be possible by caffeine. When

oocytes were incubated with caffeine, increased activity of MPF was seen along with decreased in activation of parthenogenesis and cellular fragmentation. This result indicated that caffeine can be treated to delay oocyte aging (Kikuchi et al., 2002; Kikuchi et al., 2000). Other studies also showed that caffeine treatment could prevent decreased activity of MPF and MAPK in aging bovine oocytes (Lee and Campbell, 2008). Also, when 5 mM caffeine was treated to aged mouse, the fertilization rate increased with decrease in fragmentation (Miao et al., 2009).

CHAPTER 3

DEVELOPMENTAL COMPETENCE OF QUAIL OOCYTES AFTER OVULATION

1. Introduction

In avian species, the natural fertilization oocurs shortly after the oocyte is ovulated and located in the first segment of the oviduct called infundibulum. The penetration of sperm is hindered by physical barriers. Once the oocyte enters the magnum, periviteline membrane as well and albumen secretions cover the ovum (Bakst and Howarth, 1977). Recent studies have applied the intracytoplasmic injection (ICSI) method to fertilize the oocytes from Japanese quail (*Coturnix japonica*). ICSI is a technique of microinjection of sperm in to the oocyte for fertilization and this method has been used in many species. The past studies have shown that quail oocytes collected from infundibulum and upper magnum can be successfully fertilized by injecting a single quail sperm and subsequently develop to Eyal-Giladi and Kochav (EG&K) stages II-VI (Hrabia et al., 2003; Mizushima et al., 2007). The eggs were collected from the infundibulum and magnum, within 50 minutes after ovulation, in these studies. However, the successful fertilization of the oocytes collected from other parts, isthmus and shell gland, has not been reported. If the fertilization is possible for oocytes collected from shell gland, this could become a valuable contribution for future ICSI studies. Ovum can be manually collected from shell gland by gentle abdominal massaging of the quail. If the fertilization and embryonic development is possible for oocytes located in shell gland or oviposited, no bird needs to be sacrificed for further ICSI – assisted fertilization.

Therefore, the purpose of this study was to determine the fertilization and blastodermal development rates of the oocytes collected from various hours post ovulation. From this, the maintenance of

fertilization competency after ovulation was determined. Japanese quail oocytes were collected from various time post ovulation and injected with sperm and IP_3 using ICSI method. IP_3 was injected along with sperm to improve the fertilization rate by facilitating the Ca^{2+} oscillation. Then the oocytes were cultured for 24 hours and DAPI stain was applied to the blastoderms to determine whether the oocytes were fertilized

2. Material and methods

Experimental Birds

Male and female Japanese quail (*Coturnix japonica)* with age of 6 weeks or more were kept at room temperature of 24±1℃ and photoperiod of 14L:10D (lights on at 0400 hr and off at 1800 hr). Birds were provided with free access to food and water. Female quail were caged individually for the collection of unfertilized eggs and pairs of female and male quail were caged together to obtain the fertilized eggs. Time of egg laying or oviposition was recorded for an individual quail using a video camera recoding system. Most of the female birds laid eggs consecutively for more than 5 days in a sequence between 1300 and 1700 hr. Time of ovulation for each female bird was estimated by the observation of the oviposition time. Ovulation was considered to occur within 0.5 hr to 1.5 hr after oviposition of the previous egg in the sequence. All procedures for the use and the care of animals were conducted according to a standard management program at the University Animal Farm, Seoul National University, Korea.

Oocyte Collection

For positive control, fertilized oocytes were collected from female quail that were paired with male quail hourly from 7 hr to 13 hr after preceding oviposition. For negative control, unfertilized oocytes were collected from

unpaired female quail hourly from 7 hr to 13 hr after preceding oviposition. All oocytes for control sampling were expelled from the bird's body using an abdominal massage. For the experimental group, oocyte was collected as follows: 1) for 0.5-1.5 hr after ovulation group, ova were recovered from infundibulum or upper magnum of laying quail approximately 2 hour after the preceding oviposition by surgery (n=30); 2) for 3.5-4 hr after ovulation group, ova were recovered from isthmus approximately 4 hour after the preceding oviposition by surgery (n=16); 3) for 5-6 hr after ovulation group, ova were recovered from the shell gland by gentle abdominal massage (n=11); 4) for 9-10 hr after ovulation group, ova were recovered from the shell gland by gentle abdominal massage (n=16); 5) for 22-23 rh after ovulation group, the freshly laid egg was collected (n=18).

Sperm collection and IP_3 preparation

The sexually matured male quails were separated from females. The cloaca gland of the male quails was gently squeezed to isolate semen. The semen is suspended in 1 ml of PBS and centrifuged at 1,000g for 2 min, resuspended in PBS and washing was repeated 2 more times. The washed sperm were diluted with polyvinyl-pyrrolidone (PVP) in Dulbecco Modified Eagle medium (DMEM). 100 µl of this solution was placed on a Petri dish. IP_3 was diluted to 60nM/ml and 2µl was aliquot onto the same Petri dish with the prepared sperm in PVP and DMEM. Mineral oil was added to the Petri dish until sperm and IP_3 solutions are completely covered.

Injection pipette preparation

The preparation for the injection micropipettes was same as that was described by Hrabia and Mizushima (Hrabia et al., 2003; Mizushima et al., 2010). For the ICSI injection pipette, microcapillary tube (Cat # 1-000-0500, Drummond Scientific Company, U.S.A.) was cut to half using a pipette puller (Model P-97, Sutter Instrument, U.S.A.). Then tip of the cut pipette was beveled at a 30° angle by a micropipette grinder (EG-400, NARISHIGE). The pipettes were washed with 70% ethanol and dried. Before injection, the prepared pipette was attached to a micro-manipulator and silicon oil was drawn half way into the pipette.

Intracytoplasmic sperm injection of sperm and IP_3

The microinjection procedure was same as that was described by Hrabia et al. (2003). Under an inverted microscope, IP_3 (60nM/ml) was first drawn in to a micropipette, followed by quail sperm diluted in PVP and DMEM. Around 10 sperm were picked for each injection. Under a stereomicroscope, the drawn contents were injected into the central area of the germinal disc of the oocyte after shell membrane and albumen removed.

Oocyte culture and observation of cell division

Oocytes injected with quail sperm and IP_3 were incubated in a 20-ml plastic cup filled with DMEM (pH 7.4) at 41.5℃ under 5% CO_2 for 24

hours applying the system Q1 of the surrogate culture system described by Ono (Ono et al., 1994). After the incubation, the blastoderms of the oocytes were excised and observed under a stereomicroscope for the determination of blastodermal stages according to Eyal-Giladi and Kochav stages. Then the blastoderms were fixed in ethanol-glacial acetic acid (3:1) and dehydrated by incubated in decreasing concentration of ethanol (70%, 50%, 30% and 8%) and washed with PBS twice. Then blastoderms were fixed to a glass slide and stained with 4,6-diamidino-2-phenylindole (DAPI) to determine the cell division.

3. Results

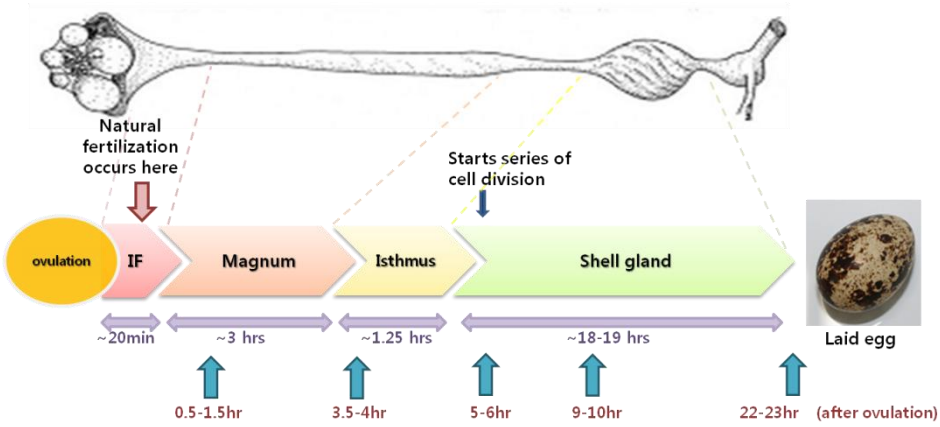
The morphology and DAPI analysis of the fertilized blastoderms and unfertilized germinal discs

As the criteria for determining the fertilization and developmental stages for the experimental groups, *in vivo* fertilized and unfertilized oocytes collected hourly at 6 to 12 hours after ovulation were subjected to DAPI analysis. As shown in Figure 2, the DAPI stained blastoderms of *in vivo* fertilized oocytes showed progressive increase in the number of the visible nuclei as the oocytes were collected at later hours after ovulation. The blastodermal morphology of the naturally fertilized oocyte collected at 6 hr post ovulation (Fig. 2A) showed the cleavage furrow near the central region. The DAPI analysis showed a few nuclei. The blaostoderm of the oocyte collected at 7 hr post ovulation (Fig. 2B) showed that the cleavage furrows were spread out in all directions and more nuclei were visible. Blastoderms excised from the oocytes collected at 8 and 9 hr post ovulation (Fig. 2C and 2D) showed that the cleavage furrows have radiated to the edge of the blastoderms. Also, the size of the blastoderms had shrunk. Increased number of nuclei could be seen from these blastoderms. The morphology of blastoderms of the oocytes collected at 10 to 12 hr post ovulation (Fig. 2E, 2F and 2G) showed much smaller blastomeres that covered the whole surface of the blastoderms. When they were DAPI stained, more nuclei were detected emitting intense blue fluorescence and they were much more distinguishable then those of the blastoderms collected at earlier hours after ovulation. However, as shown in Figure 3, the morphology of unfertilized germinal discs showed no sign of clear cleavage furrow and blastomeres. Some of them showed vacuoles

on the peripheral area of the germinal discs. No detection was found in most of the unfertilized germinal discs in DAPI analysis. DAPI detection was found in germinal discs of oocytes collected at 10 to 12 hr post ovulation. However, the size, morphology and distribution of these DAPI stained nuclei indicated that they were not true embryonic nuclei (Fig. 4E, 4F and 4G).

Intracytoplasmic sperm injection (ICSI) with quail sperm and IP_3

Applying intracytoplasmic sperm injection (ICSI) method, Japanese quail sperm and IP_3 were injected into the germinal discs of oocytes collected at 0.5-1.5, 3.5-4, 5-6, 9-10 and 22-23 hr after ovulation. After injection, oocytes were cultured for 24 hrs then the fertilization and blastodermal development were determined based on morphology observation and DAPI analysis. As shown in Table 1, the fertilization rate was highest at 60% when injection was done on oocytes collected at 0.5-1.5 hr post ovulation. 5 out of 30 oocytes injected for this group showed blastodermal development beyond EG&K stage IV based on the observation of DAPI stained embryonic nuclei and by comparing with the DAPI analysis of positive control. The fertilization was also observed in the rest of the groups (Fig. 4) although the rate had significantly dropped. No blastodermal development beyond EG&K stage IV were seen in any of the fertilized oocytes from these groups.



Time after Oviposition	2 hr.	4hr.	6hr.	10hr.	At Ovipositon
Time after Ovulation	0.5-1.5hr (30-90min)	3.5-4hr (200-240 min)	5-6hr (290-360 min)	9-10hr (530-600 min)	22-23 hr
Egg position in the Oviduct	Infundibulum(IF) to Magnum	Upper to Mid Isthmus	Shell gland	Shell gland	

Figure 1. Time of oocytes collected after ovulation and location of oocytes in the oviduct (IF: infundibulum, magnum, isthmus and shell gland) of the quail

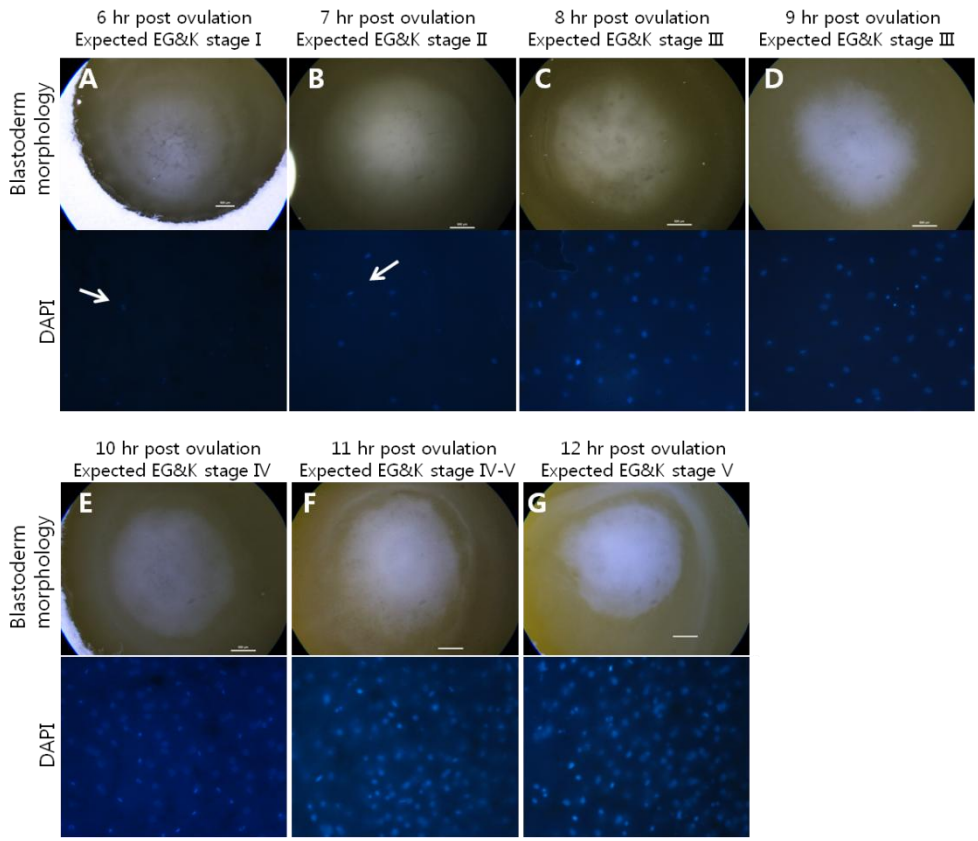


Figure 2. Morphology and DAPI stained blastoderms of *in vivo* fertilized oocytes collected at (A) 6 hr (B) 7 hr (C) 8 hr (D) 9hr (E) 10 hr (F) 11 hr to (G) 12 hr after ovulation**.** The fluorescent image shows DAPI analysis of each blastoderm located in the upper panel. White bar is 500 ㎛. DAPI photos are magnified 200X. Arrow indicates a nucleus

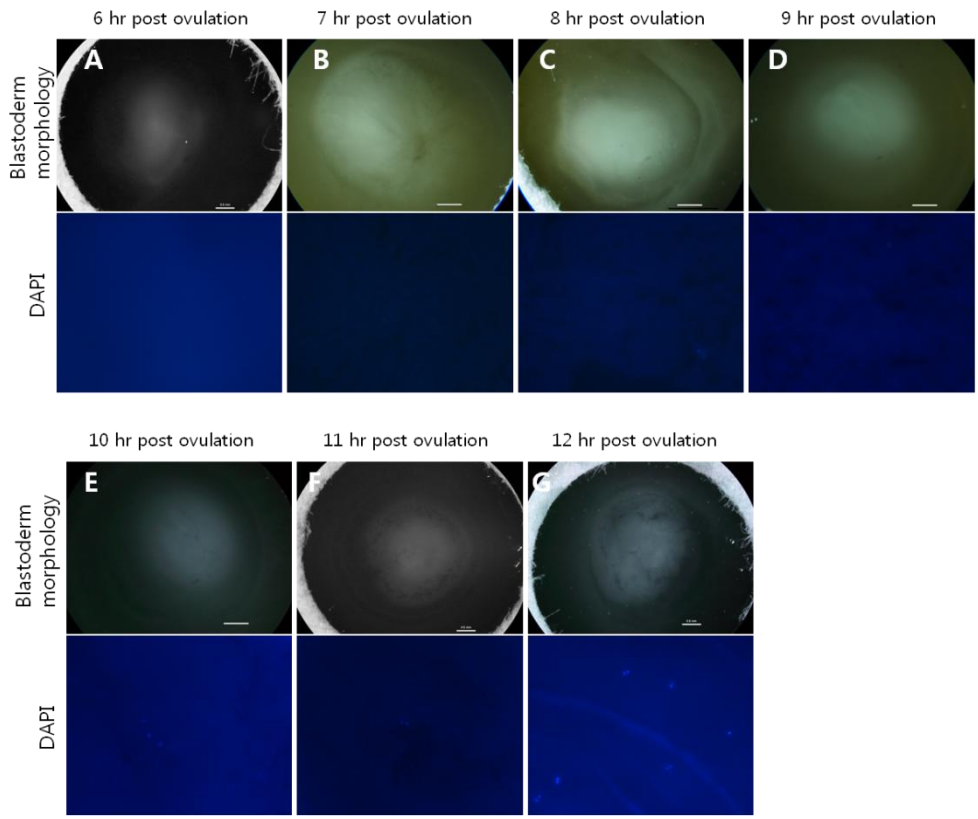


Figure 3. Morphology and DAPI stained germinal discs of unfertilized oocytes collected at (A) 6 hr (B) 7 hr (C) 8 hr (D) 9 hr (E) 10 hr (F) 11 hr to (G) 12 hr after ovulation**.** The fluorescent image shows DAPI analysis of each germinal disc located in the upper panel. White bar is 500 ㎛. DAPI photos are magnified 200X.

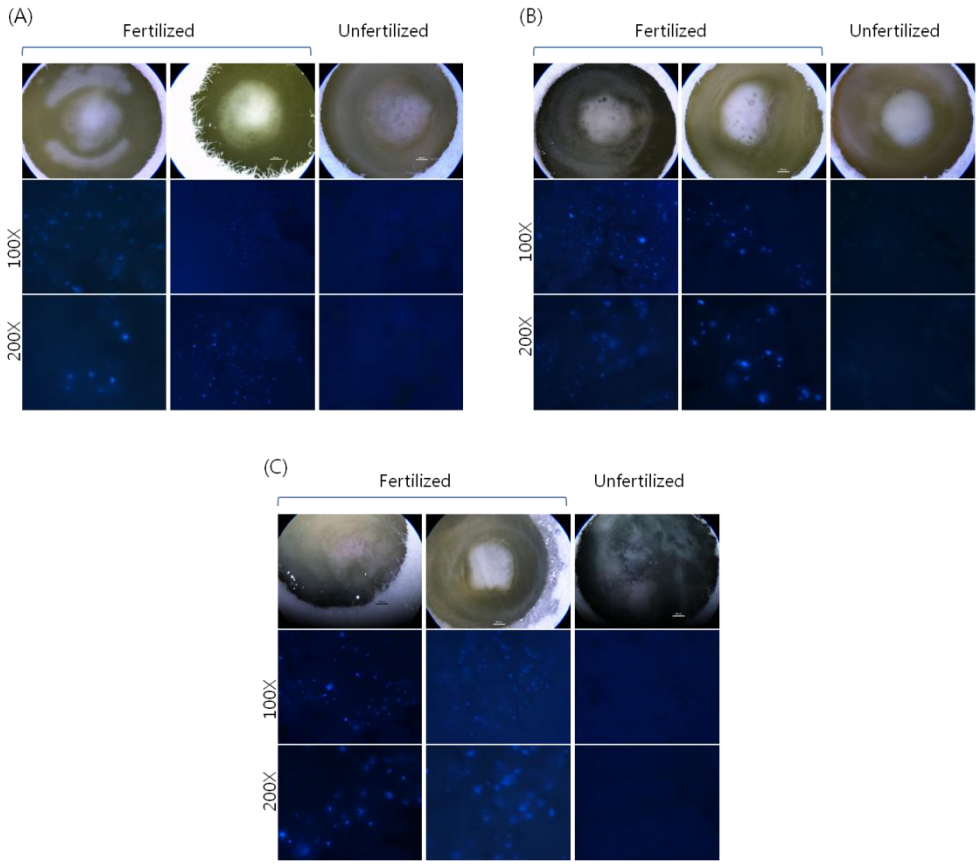


Figure 4. Morphology and DAPI analysis of ICSI performed oocytes showing fertilization and development. Oocytes were collected at (A) 5-6 hr (B) 9-10 hr and (C) 22-23 hr after ovulation for ICSI with IP_3 followed by 24 hr culture in DMEM. The right part of each panel also shows the unfertilized germinal discs of ICSI performed oocytes.

Table 1 Fertilization and development rates of ICSI assisted quail oocytes collected at various hours after ovulation. Each oocyte was injected with quail sperm and IP_3

Experimental group	1	2	3	4	5
Time after Ovulation	0.5-1.5 hr	3.5-4 hr	5-6 hr	9-10 hr	22-23 hr
General Oocyte position in the oviduct	IF to mid magnum	Isthmus	Shell gland	Shell gland	Oviposited
Fertilization Rate* (%)	18/30 **60%**	5/16 **31.3%**	4/11 **36.4%**	3/16 **18.8%**	5/18 **27.8%**
Development beyond EG&K stage IV (%)	5/30 **16.7%**	0/16 **0%**	0/11 **0%**	0/16 **0%**	0/6 **0%**

* Fertilization rate was determined by the morphology and number of nuclei detected in DAPI analysis of each blastoderm

4. Discussion

This is the first report demonstrating that the oocyte in the shell gland is capable of fertilization in Japanese quail, although fertilization rate is low (20-30%) when compared to that of oocyte in the magnum (60%). Previous reports showed the successful fertilization and subsequent blastoderm development when ICSI was performed with sperm in oocytes from infundibulum and magnum. Around 13-16% of the oocytes were fertilized and developed to Eyal-Giladi and Kochav (EG&K) stages II-VII (Hrabia et al., 2003). Micro-injection of ejaculated and testicular sperm into the oocyte from infundibulum induced the fertilization and embryonic development (EG&K stages IV-VII) in 22.6 and 20% of the quail oocyte, respectively (Takagi et al., 2007). Recently, the fertilization rate improved to more than 40% and developmental stage of IV-VI in the oocytes from the infundibulum or magnum when injected with round spermatid and PLCzeta cRNA (Mizushima et al., 2009). In the current study, the fertilization rate improved to 60% when oocytes collected from infundibulum or magnum were injected with sperm along with IP_3.

Oocytes can be maintained until the oviposition of the egg, at 22 to 23 hr after ovulation. First the oocytes collected at different hours post ovulation were injected with quail sperm and IP_3. The positive sign for fertilization and embryonic development was determined by the observation of the morphology and DAPI staining of the blastoderms. Some of the fertilized oocytes showed the morphology of the blastoderms at different stages described by Eyal-Giladi and Kochav (Eyal-Giladi and Kochav, 1976). The cleavage furrow formation and blastomeres were clearly shown in some of the fertilized blastoderms. The DAPI staining of these blastoderms confirmed the cell division by the DAPI detected

embryonic nuclei, The size, morphology and the number of DAPI detected embryonic nuclei were used as criteria to determine the embryonic stages (Perry, 1987). The morphology of the fertilized blastoderms of oocytes collected from isthmus, shell and freshly laid eggs showed the progressive increase in number of vacuoles as the oocyte was collected at later hours post ovulation. This may be an indication of cellular fragmentation often seen in aged oocytes (Miao et al., 2009).

The significant decrease in the fertilization and developmental rates were seen when ICSI was performed in oocytes collected from isthmus, shell gland and freshly laid eggs. This result indicates that the oocytes in the infundibulum and upper magnum, which is within 90 minutes after ovulation, are in the optimal condition for the successful fertilization and subsequent embryonic development. It seems that oocytes start to lose the fertilization and developmental capability as they enter isthmus at about 3.5-4 hr post ovulation. There may be certain environmental and chemical factors within the oviduct acting on the oocytes to deteriorate the capability of fertilization and embryonic development. In the magnum, the oocytes obtain the albumen secreted from tubular cells in the magnum mucosa (Moran, 1987). These egg white proteins have acidic isoelectric points (Stevens, 1991). According to the *ex ovo* surrogate shell culture system of chicken and quail oocyte, the constant pH of 7.2-7.4 and temperature 41.5℃ are crucial for the successful embryonic development (Ono et al., 2005). The pH and the chemical environment of the oocyte change once the oocyte is surrounded by the secretions in the magnum. These factors may be negatively acting on the oocyte to lower the fertilization and embryonic developmental rates. Also, it has been reported in chicken oocyte the extraembryonic maternal RNA is degraded during the 24 hr incubation in the oviduct until the

oocyte is oviposited. This is shown by the 80% decrease in the content of the extraembryonic RNA after oviposition. It was reported that RNase acitivity may be causing this degradation (Maleszewski et al., 1999). In addition, the high level of DNase I and II activities has been reported in the quail oocyte and it has been suggested that DNase I and II may play a role in removal of supernumerary sperm in birds (Stepinska and Olszanska, 2003). In the current study, a single sperm was injected with IP_3 to mimic the effect of the polyspermy. Therefore, DNase I and II were not necessary in the process of degrading the extra sperm. Although the specific mechanisms of DNase I and II have not been discovered, they may also participate in lowering the fertilization and embryonic development rates as the oocyte is remained unfertilized. As the oocyte is collected at later time after ovulation, the oocyte encounters more environmental changes such as changes in pH, temperature and prolonged activity of enzymes such as RNase. These environmental and chemical factors may participate in lowering the fertilization and embryonic developmental rates of the oocytes located in isthmus and beyond.

In conclusion, our results indicate that quail oocytes are able to maintain the fertilization capability until the egg is laid. The time period for the optimal fertilization and embryonic development is within 30 to 90 minutes post ovulation, while the oocytes are located in the infundibulum and upper magnum. Once the oocyte enters the isthmus, the fertilization rate significantly decreases and the embryonic development ceases shortly after fertilization. However, additional treatment, such as caffeine, may recover to raise the development as shown in mammals (Kikuchi et al., 2000; Lee and Campbell, 2008). If the fertilization and development rates can be improved by these treatments in quail, the oocytes from shell gland can retrieved by gentle abdominal massage and used for future ICSI studies. This could solve the ethic issue of sacrificing the birds and further

contributes to conserving endangered avian species.

CHAPTER 4

MOLECULAR CHARACTERIZATION OF QUAIL OOCYTES AFTER OVULATION

1. Introduction

It has been well known in mammals that the fertilization is characterized by the increased in the intracellular level of Ca^{2+} in the cytoplasm of the oocyte. This event is triggered when the spermatozoon interacts with the zona pellucida, an extracellular coat, of the oocyte (Ben-Yosef and Shalgi, 1998). The fertilizing sperm undergoes an acrosome reaction which allows the sperm to penetrate the zona pellucida to interact with the plasma membrane of the egg (Schultz and Kopf, 1995). This interaction between the spermatozoon and oocyte triggers the oocyte activation and fertilization. Mammalian fertilization normally occurs when the oocyte is in the metaphase arrest of the second meiotic division of the cell cycle. This metaphase arrest is regulated by the high level of Maturation promoting factor (MPF) and Mitogen-activating protein kinase (MAPK) (Tunquist and Maller, 2003). Cyclin B, the regulatory unit of MPF must be continuously synthesized in order to keep the high level of MPF (Jones, 2004). When a fertilizing spermatozoa enters the oocyte to release the sperm factor, IP_3 binds to its receptors in endoplasmic reticulum membrane opens the Ca^{2+} channel which raise the Ca^{2}+ concentration in the cytoplasm triggering the waves of Ca^{2+}. Free Ca^{2+} ions in the cytoplasm bind to the calmodulin to activate CaMKII (Markoulaki et al., 2003), which in turn degrades emi to activate anaphase promoting factor (APC). Cyclin B is ubiquitinated by the activated APC and the level of MPF decreases. This allows the oocyte to be released from the metaphase II arrest and continue with cell cycle (Tripathi et al., 2010)

The fertilizing spermatozoon enters the oocytes to release sperm factor, which trigger the release of Ca^{2+} from ER. Free Ca^{2+} ions in the

cytoplasm bind to the calmodulin to activate CaMKII (Markoulaki et al., 2003). This causes emi degradation which in turn activates anaphase promoting factor (APC). The activated APC ubiquitinates cyclin B. This lowers the level of MPF and the oocyte is released from the metaphase II arrest and meiosis resumption occurs (Miyazaki et al., 1993; Tripathi et al., 2010).

Mammalian oocytes have an optimal window of period for fertilization to occur. It has been reported that the optimal fertilization period for mouse and rat is less than 12 hours and 14 hrs after the oocyte is ovulated, respectably (Miao et al., 2009). The process called oocyte aging occurs when the fertilization delayed. Mammalian oocyte aging has been characterized with low fertilization rate (Goud et al., 1999) and abnormal embryonic development (Tarin et al., 1999). It is also associated with the premature decrease in the level of MPF and MAPK (Tian et al., 2002). When an aged oocyte is activated or fertilized the abnormal calcium oscillation can induce apoptosis and cellular fragmentation (Gordo et al., 2002). Pro-apoptotic factors such as caspase and cytochrome C are known to be elevated and the expression anti-apoptotic BCL2 is reported to be down-regulated in aged oocyte (Wilding et al., 2001). The recent studies on mammalian oocyte aging focus on reversing the aging process to improve the fertilization and embryonic development (Lee and Campbell, 2008; Miao et al., 2009).

However, the optimal window of fertilization period and the mechanisms of oocyte aging process for avian species have not been established yet. Understanding these processes will be a crucial contribution towards studies on avian model system. In this study, we have selected genes that are known to be involved in oocyte activation,

fertilization and aging. Quantitative RT-PCR analysis was performed to observe the differences in gene expressions between aged and fresh oocytes in both unfertilized and IP_3 injected groups.

2. Material and methods

Oocyte collection

Unfertilized ova were retrieved from unpaired female quail at 2 hour after preceding oviposition. For the IP_3 injection group, unfertilized ova were collected from unpaired female quail at 2 hour, 6 hour after previous oviposition. Also, freshly laid eggs used for 22-23hr group.

Intracytoplasmic sperm injection of IP_3

The microinjection procedure was same as that was described by Hrabia (Hrabia et al., 2003) and Mizushima (Mizushima et al., 2010). Under an inverted microscope, IP_3 (60nM/ml) was drawn in to a micropipette. Under a stereomicroscope, the drawn contents were injected into the central area of the germinal disc of the oocyte after shell membrane and albumen removed.

Oocyte culture

Both unfertilized and IP_3 injected oocytes were incubated in a 20-mL plastic cup filled with DMEM (pH 7.4) at 41.5℃ under 5% CO_2 for 3 hours.

Real time PCR

Unfertilized quail oocytes at (1) 0.5-1.5 (2) 5-6 (3) 22-23 hr after ovulation were collected for injection group. Unfertilized oocytes at 0.5-1.5 hr post ovulation were collected for negative control group. Oocytes for injection group were injected with IP_3 using ICSI method. After the oocytes were cultured in DMEM inside a CO_2 incubator for 3 hours, three blastodiscs were excised for total RNA extraction by TRIzol reagent (Invitrogen). 0.2 µg of total RNA from three blastodiscs was used to synthesize single-stranded cDNA according to manufacturer's protocol of the Superscript III First-Strand Synthesis System (Invitrogen). The synthesized cDNA was diluted 5-fold. The program Primer 3 (http://frodo.wi.mit.edu/) was used to design the primers for the selected genes (Table 1). The real-time PCR was performed with EvaGreen (Biotium). The each 20 µl of PCR mixture contained 2 µl of cDNA, 2 µl of 10x PCR buffer, 1.6 µl of 2.5 mM dNTP mixture, 1 µl of 10 pmol reverse and forward primer each, 1 µl of 20X EvaGreen and 0.2 µl of Taq DNA polymerase. The PCR conditions were 94 ℃ for 3 min followed by 40 cycles at 94 ℃ for 30 sec, 60 ℃ for 30 sec and 72 ℃ for 30 sec, using a melting curve program (increase in temperature from 55 ℃ to 95 ℃ at a rate of 0.5 ℃ per 5 sec). Each sample was performed in triplicate. The results from real-time PCR were normalized relative to the expression of *GAPDH* and calculated using the $2^{-\Delta\Delta Ct}$ Method.

3. Results

Relative expression of genes associated with IP_3 receptors for calcium release

To examine the relative expressions of *ITPR1, ITPR2* and *ITPR3,* the known receptor for IP_3 the total RNA was extracted from each blastoderm or germinal disc of unfertilized and IP_3 injected oocytes that were retrieved from various hours post ovulation. Quantitative RT-PCR was performed to compare the relative expressions of *ITPR1, ITPR2* and *ITPR3* in unfertilized 0.5-1.5h post ovulation group and IP_3 injected group (Fig. 1). For *ITPR1* and *ITPR2,* the expression was higher in IP_3 injected 0.5-1.5h post ovulation than the oocytes collected at later hour groups (Fig. 1A and 1B). The higher expression of IP_3 injected group when compared to the negative control. On the other hand, for *ITPR3,* the higher expression was observed in 22-23h IP_3 injected oocytes (Fig. 1C). Similar expression level was seen for negative control and IP_3 injected 0.5-1.5h post ovulation oocytes.

Relative expression of genes associated with anti-apoptosis and pro-apoptosis

For the expression pattern analysis of genes associated with apoptosis in aging oocytes, the expression level between unfertilized, as negative control, and IP_3 injected oocytes were observed for *BCL2L1* and *CYCS* (Fig. 2). For both genes, significantly higher expression was observed in IP_3 injected oocytes when compared to the unfertilized oocyte.

For *BCL2L1*, the expression level was lower in IP_3 injected oocytes collected at 5-6h and 22-23h when compared to 0.5-1.5h post ovulation (Fig. 2A). For *CYCS*, although the expression level in IP_3 injected oocytes at 5-6h and 22-23h post ovulation were higher than the negative control, it was lower than the IP_3 injected oocyte at 0.5-1.5h post ovulation (Fig. 2B).

Relative expression analysis of genes associated with progression of cell cycle in oocyte

To analyze the expression pattern of the selected genes that may be involved in release of metaphase II arrest and resumption of meiosis in oocyte, the Quantitative RT-PCR was performed with each blastoderm and germinal disc isolated from unfertilized oocytes and IP_3 injected oocytes collected at various hours after ovulation. For all three genes, *CDK1*, *MAPK1* and *MAD2L2*, significantly higher expression could be observed when IP_3 was injected into an oocyte collected 0.5-1.5h post ovulation (Fig. 3). However, the expressions were much lower in IP_3 activated oocytes collected at later hours for *CDK1* and *MAD2L2* (Fig. 3A and 3C). On the other hand, the *MAPK1* expression rises for oocyte collected at 22-23h post ovulation in the injection group (Fig. 3B).

Relative expression analysis of genes associated with stemness, germness and microRNA processing

For the observation of presence of the gene activity of genes associated with stemness, germness and micro RNA processing in the very early stage of the oocyte activation, the relative expression patterns for *OCT4*, *NANOG*, *DDX4*, *PIWIL1*, *DICER* and *DROSHA* were analyzed

(Fig. 4, 5 and 6). The expressions for all six genes showed very weak or no activity of genes genes in the unfertilized oocytes collected at 0.5-1.5h post ovulation. Higher expression of genes, except *PIWIL1*, were observed for 5-6h post ovulation for IP_3 injected oocytes when compared to the oocytes collected 0.5-1.5h and 22-23h ovulation for IP_3 injection. On the other hand, for *PIWIL1* gene, the higher expression was observed in oocytes collected at 0.5-1.5h post ovulation than the oocytes collected at later hours for IP_3 injection (Fig. 5B).

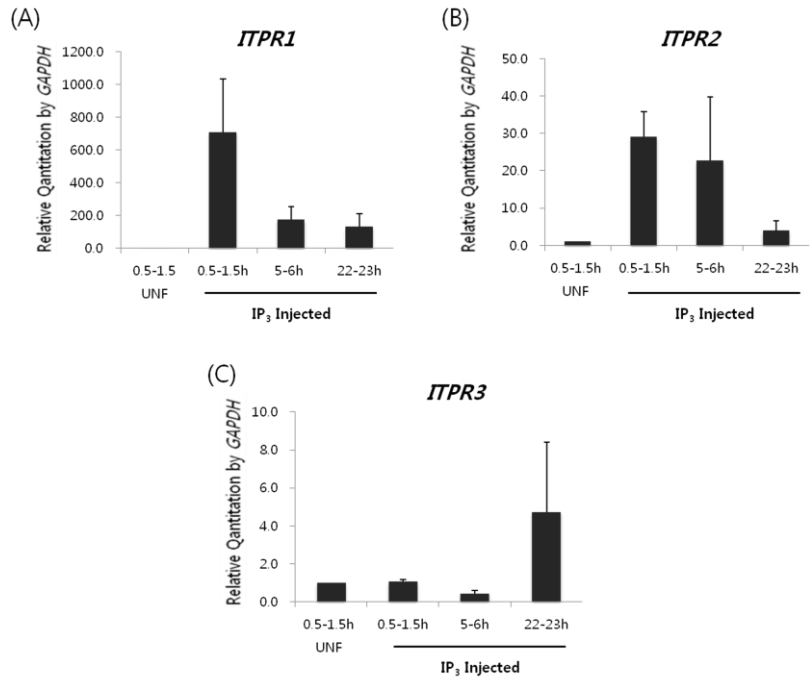


Figure 1. Quantitative RT-PCR analysis of genes presumably associated with calcium release, (A) *ITPR1* (B) *ITPR2* (C) *ITPR3* in unfertilized and IP_3 injection oocytes. The analysis was normalized to control expression of *GAPDH.*

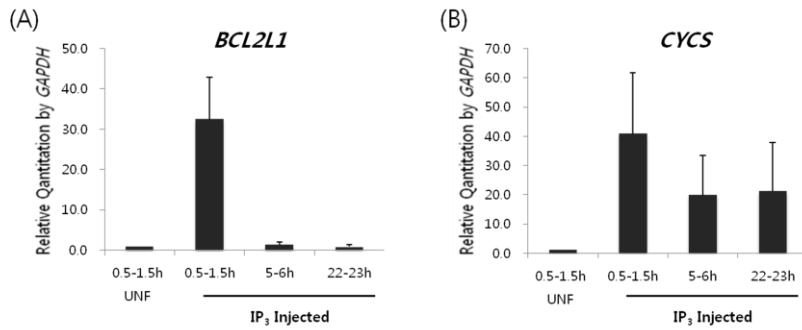


Figure 2. Quantitative RT-PCR analysis of genes presumably associated with apoptosis, (A) *BCL2L1* (B) *CYCS* in unfertilized and IP_3 injection oocytes. The analysis was normalized to control expression of *GAPDH.*

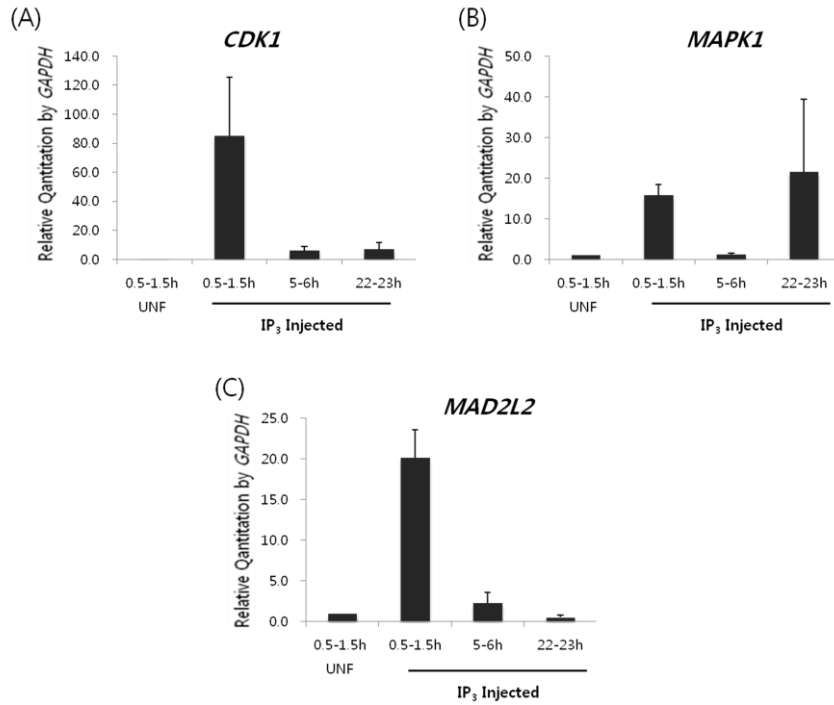


Figure 3. Quantitative RT-PCR analysis of genes presumably associated with oocyte cell cycle progression in (1) *CDK1* (2) *MAPK1* (3) *MAD2L2* in unfertilized and IP_3 injection oocytes. The analysis was normalized to control expression of *GAPDH.*

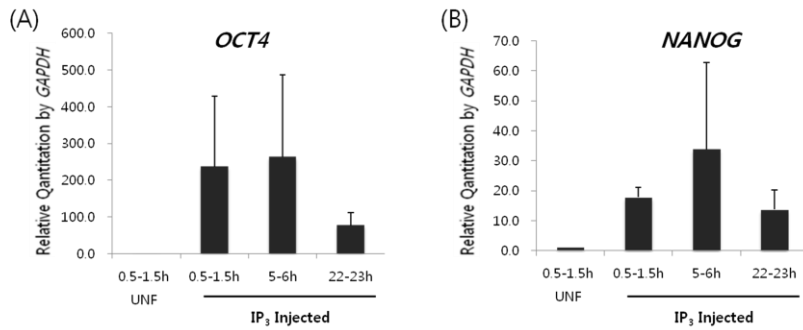


Figure 4. Quantitative RT-PCR analysis of genes presumably associated with stemness, (A) *OCT4* (B)*NANOG* in unfertilized and IP_3 injection oocytes. The analysis was normalized to control expression of *GAPDH.*

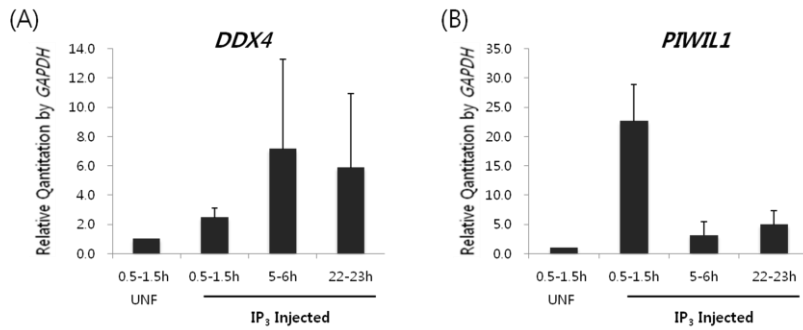


Figure 5. Quantitative RT-PCR analysis of genes presumably associated with germness, (A) *DDX4* (B) *PIWIL1* in unfertilized and IP_3 injection oocytes. The analysis was normalized to control expression of *GAPDH.*

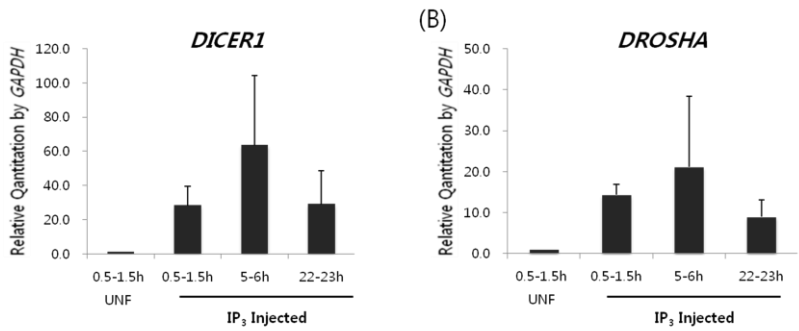


Figure 6. Quantitative RT-PCR analysis of genes presumably associated with microRNA processing, (A) *DICER1* (B) *DROSHA* in unfertilized and IP_3 injection oocytes. The analysis was normalized to control expression of *GAPDH.*

Table 1 Information of the primer sequences used for quantitative RT-PCR analysis

Gene name	Description	Accession No.	Primer Sequence (5' → 3')	
			Forward	Reverse
ITPR1	inositol 1,4,5-trisphosphate receptor, type 1	NM_001174059.1	TGAGGAAGATGACCAGGAGTG	CAGGCACAGACACCAGAGAG
ITPR2	inositol 1,4,5-trisphosphate receptor, type 2	XM_001235612.2	CCCTTTGGAGATGATGGAGA	GGGCTTGGAGATGAAAAACA
ITPR3	inositol 1,4,5-trisphosphate receptor, type 3	XM_418035.3	CGAGACGGAGAACAAGAAGG	CCAGAAGGGTTGGATGAAGA
CDK1	cyclin-dependent kinase 1	NM_205314.1	AAGATAGAGAAGATTGGGGAAGG	GCATAAGAACATCCTGAAGACAGA
MAPK1	mitogen-activated protein kinase 1	NM_204150.1	ACTCAACACCTCAGCAACGA	AGCCAGTCCGAAGTCACAAA
MAD2L2	mitotic arrest deficient-like 2	NM_001025578.1	AATCACCCAGCCACCTCTC	TGTAGCAGCCTCTCGTGTGT
BCL2L1	BCL2-like 1	NM_001025304.1	GCTTTCAGCGACCTCACCT	TACCCGCATCTCCTTGTCC
CYCS	cytochrome c, somatic	NM_001079478.1	TGGACGCAAAACAGGACA	CTTCTTCTTGATACCCGCAAA
POUV	POU domain class 5 transcription factor 1	NM_0011101781	TGAAGGGAACGCTGGAGAGC	ATGTCACTGGGATGGGCAGAC
NANOG	nanog homeobox	NM_001146142.1	AACTCTGCGGGGCTGTCTTG	AAAAGTGGGGCGGTGAGATG
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	NM_204708.1	CCTTGCAGCCTTTCTTTGTC	ACGACCAGTTCGTCCAATTC
PIWIL1	piwi-like 1 (Drosophila)	NM_001098852.1	CCGAAATGGAGAAGATGTGAGGA	TGTGATTAGGGATGCTGACTGGG
DICER1	dicer 1, ribonuclease type III	NM_001040465.1	AGCAAGGCTGTTGAAGAGGA	GTTCTCTCCAGTCGCACACA
DROSHA	drosha, ribonuclease type III	NM_001006379.1	GGGGGACCAGGAGCAAGAAC	GGATTTTGTGTGGAAGGGGGA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_204305.1	CACACAGAAGACGGTGGATG	AACAGAGACATTGGGGGTTG

4. Discussion

This is the first study to observe the mRNA expression changes among the quail oocytes collected from different parts of oviduct. The result demonstrated that low mRNA expression of *ITPR1* and *ITPR2* may be associated with the low fertilization rate in oocytes collected from shell gland and oviposited eggs, hence, participate in the process of oocyte aging. The mRNA expression level was compared between the unfertilized oocytes at 0.5-1.5 hr for negative control and oocytes collected at 0.5-1.5hr, 5-6 hr and 22-23 hr post ovulation that were IP_3 injected. The oocytes at 0.5-1.5 hr post ovulation showed the significantly higher responsiveness to IP_3 when compared to oocytes from later hours. The expression gradually decreased as the oocytes were collected from the later hours.

The result of this study indicates that the mRNA expression of IP_3 receptors, specifically *ITPR1* and *ITPR2*, is decreased with the aging process of quail oocyte. It may be possible that the IP_3 receptors are desensitized or functionally disrupted in the aged oocytes, causing abnormal rise in Ca^{2+} transient when IP_3 is injected. This is in accordance with the studies done in mammalian aged oocyte. It has been reported that the Ca^{2+} releasing mechanism of the oocyte is gradually lost following ovulation until IP_3 can no longer trigger Ca^{2+} oscillation in aged mouse oocyte. Therefore, Ca^{2+} homeostasis is optimal in freshly ovulated oocyte and then diminishes with the aging process (Jones et al., 1995). Another study showed that aged mouse oocyte could not sustain long-lasting Ca^{2+} oscillation and this disruption in Ca^{2+} oscillation initiated programmed cell death rather than normal oocyte activation (Gordo et al., 2002). Other studies reported that the phosphorylation of IP_3 receptors, especially type 1

in mammals, is in a close association with cell cycle related kinases such as MAPK. The phosphorylation of these kinases serves to optimize Ca^{2+} release as a regulatory mechanism of IP_3 receptors (Jellerette et al., 2004; Lee et al., 2006a). However, activities of these meiotic kinases are reported to be prematurely inactivated in the aged oocytes in comparison to fresh oocytes (Miao et al., 2009). These reports indicate that the reduced activities of MPF and MAPK may be inhibiting the function of IP_3 receptors, therefore, disrupting the Ca^{2+} release in aged oocytes. The more recent study has shown that the caspase-3-mediated truncation of IP_3 receptors causes the Ca^{2+} to leak out from the ER. Normally, this Ca^{2+} leakage is compensated by enhanced Ca^{2+} uptake activity. However, Ca^{2+} uptake activity is lost in aged mouse oocyte as the cellular metabolism is compromised. This induces the forward-feedback mechanism to trigger apoptosis in the oocyte (Verbert et al., 2008). These studies show that the functional disruption of IP_3 receptors may be responsible for the abnormal Ca^{2+} transient in aged oocyte. Collectively, this disruption in Ca^{2+} homeostasis is negatively affecting the fertilization and embryonic development in the aging process by triggering apoptosis and cellular fragmentation (Miao et al., 2009). In addition, studies done in bovine oocytes showed that although the all three isoforms of IP_3 receptors were expressed in metaphase II oocytes. Specifically type 1 IP_3 receptors were present in large amounts. These studies suggested that type 1 of the IP_3 receptors may be the one responsible for majority of Ca^{2+} release at fertilization (He et al., 1999). No significant difference in the expressions of *ITPR3* between unfertilized oocytes and IP_3 injected oocytes observed in the present study may also suggest that although IP_3 receptor type 3 are present in the oocyte, its main role may not be involved with Ca^{2+} release during oocyte activation and fertilization.

The similar tendency of gene expression was observed for *BCL2L2*,

CDK1 and *MAD2L2* genes in the oocyte. *BCL2* is an anti-apoptotic factor that is also known to interact with the IP_3 receptors to regulate IP_3 medicated Ca^{2+} release from the endoplasmic reticulum. During fertilization and oocyte activation, *BCL2* pays a dual role of promoting prosurvival Ca^{2+} signaling and at the same time inhibiting proapoptotic Ca^{2+} signals (Distelhorst and Bootman, 2011). C*DK1* is the cdc2 kinase subunit forming a maturation promoting factor (MPF) along with the regulatory subunit cyclin B (Madgwick and Jones, 2007). The high level of MPF maintains the matured oocyte in the metaphase II arrest. MAD2 is an mitotic or meiotic spindle checkpoint protein. It has been reported that the premature degradation of BCL2, MPF and MAD were observed in mammalian oocytes undergoing aging process (Ma et al., 2005). Reduced level of Bcl-2 protein in aged oocytes promotes the fertilizing Ca^{2+} signal into an apoptosis inducing signal (Gordo et al., 2002). Low activity of MAD2 in aging oocyte fails to inhibit anaphase-promoting complex (APC) to ubiquinate the target protein. This in turn induces premature degradation of MPF. Also, the reduced activity of MAD2 no longer able to check abnormal chromosomes in the oocytes (Ma et al., 2005). Therefore, the disruption and degradation of these factors likely lead to low fertilization, abnormal embryonic development, apoptosis and cellular fragmentation of the mammalian oocytes. According to the results of the present study, significantly lower expression of *BCL2L1*, *CDK1* and *MAD2L2* were observed in the oocytes collected at later hours post ovulation when compared to the oocytes collected within 0.5-1.5h post ovulation. These observations may indicate that these genes may also play an important role in IP_3 induced Ca^{2+} oscillation and their functions may be disrupted in the aged oocytes in quails.

The expression of the genes involved in stemness, germness and microRNA processing were significantly higher in IP_3 injected oocytes

than the negative control oocytes which may suggest that their involvement in IP_3 mediated oocyte activation. However, their expressions of the aged oocytes were inconsistent. This may indicate that they may not be involved in the aging process of the quail oocytes.

In conclusion, our results indicate that the genes coding for IP_3 receptors, *ITPR1* and *ITPR2*, and also *BCL2L1*, *CDK1* and *MAD2L2* may be involved in the aging process, causing decreased rate of fertilization and embryonic development in quail oocytes collected from shell gland and oviposited eggs. Discovering the ways to maintain the function and sensitivity of IP_3 receptors in aged oocytes could trigger the normal Ca^{2+} release from the ER. This may contribute to improve the fertilization and embryonic development rates in aged quail oocytes.

CHAPTER 5

GENERAL DISCUSSION

To determine the maintenance of fertilization capability of quail oocytes, ICSI was used to fertilize the oocytes collected at various hours post ovulation. After determining the fertilization rate of the oocytes collected from various hours post ovulation, some of the genes were selected to see the quantitative expression of them. This part of the study was done to determine which genes may be involved in the low fertilization rate of the aged oocytes by observing the differences in gene expressions between aged and fresh oocytes.

In the first study, the maintenance of fertilization and developmental capability in Japanese quail were determined. The oocytes were collected from 0.5-1.5 hr, 3.5-4 hr, 5-6 hr, 9-10 hr and 22-23 hr post ovulation and performed ICSI with quail sperm and IP_3 to facilitate fertilization. The injected oocytes were then cultured for 24 hrs in CO_2 incubator. Germinal discs or blasotoderms were excised for the observation of fertilization and cell division by DAPI stain. The result shows oocytes collected 0.5-1.5 hr post ovulation had a highest fertilization and developmental rate of 60% and 16.7%, respectively. The fertilization was possible in other oocytes collected at more delayed time. However, the fertilization rate drops significantly and the blastodermal development beyond EG&K stage was not observed. The result indicates that quail oocyte is in its optimal condition to be fertilized within 1.5 hr post ovulation. Also, the fertilization was possible in aged oocytes, the development is ceased short time after the fertilization occurred.

In the second part of the study, selected genes were analyzed by quantitative RT-PCR to compare the expressions between oocytes collected at 0.5-1.5 hr, 5-6 hr and 22-23 hr post ovulation. Collected oocytes were injected with IP_3 using ICSI to trigger Ca^{2+} oscillation.

Unfertilized oocytes collected at 0.5-1.5 hr after ovulation were also analyzed. After 3 hour incubation in CO_2, the blastoderms and germinal discs of the oocytes were isolated for total RNA extraction. Three germinal discs or blastoderms were used for the RNA extraction. After cDNA was synthesized, real-time PCR was performed with EvaGreen. The expressions were normalized with *GAPDH*. The genes selected to be analyzed were known to be involved in Ca^{2+} oscillation, cell cycle regulation, apoptosis, stemness, germness and processing of microRNA in mammalian oocyte. Most of the genes were upregulated in the oocytes collected at 0.5-1.5 hr post ovulation compared to the aged oocytes in IP_3 injected group. This indicates the high responsiveness to IP_3 in oocytes from infundibulum and magnum. However, the expression of genes such as CYCS, MAPK1 and genes associated with stemness, germness and microRNA processing were inconclusive. Significantly higher expression of *ITPR1, ITPR2, CDK1, MAD2L2 and BCL2L1* were observed in unfertilized oocytes from 0.5-1.5 hr post ovulation when compared to oocytes from later hours. Therefore, the low mRNA expression of these genes may be involved in the low fertilization rate in aged oocytes.

Finally, the fertilization competency of the quail oocytes were observed until the egg is oviposited although the fertilization rate and developmental rate are decreased in the oocytes from isthmus, shell gland and laid eggs. Also, IP_3 receptors genes found to be associated with the low fertilization in aged quail oocytes. This study was the first step towards determining the fertilization capability in birds and to apply the new findings to contribute towards solving the ethical issue of sacrificing birds and conserving endangered avian species.

REFERENCES

Adenot, P. G., Szollosi, M. S., Geze, M., Renard, J. P., and Debey, P. (1991). Dynamics of paternal chromatin changes in live one-cell mouse embryo after natural fertilization. *Mol Reprod Dev* **28**, 23-34.

Alak, B. M., Smith, G. D., Woodruff, T. K., Stouffer, R. L., and Wolf, D. P. (1996). Enhancement of primate oocyte maturation and fertilization in vitro by inhibin A and activin A. *Fertil Steril* **66**, 646-53.

Almeida, E. A., Huovila, A. P., Sutherland, A. E., Stephens, L. E., Calarco, P. G., Shaw, L. M., Mercurio, A. M., Sonnenberg, A., Primakoff, P., Myles, D. G., and White, J. M. (1995). Mouse egg integrin alpha 6 beta 1 functions as a sperm receptor. *Cell* **81**, 1095-104.

Bakst, M. R., and Howarth, B., Jr. (1977). Hydrolysis of the hen's perivitelline layer by cock sperm in vitro. *Biol Reprod* **17**, 370-9.

Bekhtina, V. G. (1960). [The early stages of cleavage in the chick embryo]. *Arkh Anat Gistol Embriol* **38**, 77-85.

Ben-Yosef, D., and Shalgi, R. (1998). Early ionic events in activation of the mammalian egg. *Rev Reprod* **3**, 96-103.

Berridge, M. J. (1990). Calcium oscillations. *J Biol Chem* **265**, 9583-6.

Carroll, J., Swann, K., Whittingham, D., and Whitaker, M. (1994). Spatiotemporal dynamics of intracellular [Ca2+]i oscillations during the growth and meiotic maturation of mouse oocytes. *Development* **120**, 3507-17.

Chousalkar, K. K., and Roberts, J. R. (2008). Ultrastructural changes in the oviduct of the laying hen during the laying cycle. *Cell Tissue Res* **332**, 349-58.

Christians, E., Davis, A. A., Thomas, S. D., and Benjamin, I. J. (2000). Maternal effect of Hsf1 on reproductive success. *Nature* **407**, 693-4.

Cisek, L. J., and Corden, J. L. (1989). Phosphorylation of RNA polymerase by the murine homologue of the cell-cycle control protein cdc2. *Nature* **339**, 679-84.

Clute, P., and Pines, J. (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat Cell Biol* **1**, 82-7.

Collas, P., and Poccia, D. (1998). Remodeling the sperm nucleus into a male pronucleus at fertilization. *Theriogenology* **49**, 67-81.

Distelhorst, C. W., and Bootman, M. D. (2011). Bcl-2 interaction with the inositol 1,4,5-trisphosphate receptor: role in Ca(2+) signaling and

disease. *Cell Calcium* **50**, 234-41.

Ducibella, T., Schultz, R. M., and Ozil, J. P. (2006). Role of calcium signals in early development. *Semin Cell Dev Biol* **17**, 324-32.

Evsikov, A. V., Graber, J. H., Brockman, J. M., Hampl, A., Holbrook, A. E., Singh, P., Eppig, J. J., Solter, D., and Knowles, B. B. (2006). Cracking the egg: molecular dynamics and evolutionary aspects of the transition from the fully grown oocyte to embryo. *Genes Dev* **20**, 2713-27.

Evsikov, A. V., and Marin de Evsikova, C. (2009). Gene expression during the oocyte-to-embryo transition in mammals. *Mol Reprod Dev* **76**, 805-18.

Eyal-Giladi, H., and Kochav, S. (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev Biol* **49**, 321-37.

Fan, H. Y., and Sun, Q. Y. (2004). Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biol Reprod* **70**, 535-47.

Fissore, R. A., Longo, F. J., Anderson, E., Parys, J. B., and Ducibella, T. (1999). Differential distribution of inositol trisphosphate receptor isoforms in mouse oocytes. *Biol Reprod* **60**, 49-57.

Foltz, K. R., and Shilling, F. M. (1993). Receptor-mediated signal transduction and egg activation. *Zygote* **1**, 276-9.

Fulka, J., Jr., Jung, T., and Moor, R. M. (1992). The fall of biological maturation promoting factor (MPF) and histone H1 kinase activity during anaphase and telophase in mouse oocytes. *Mol Reprod Dev* **32**, 378-82.

Gao, Z., and Garbers, D. L. (1998). Species diversity in the structure of zonadhesin, a sperm-specific membrane protein containing multiple cell adhesion molecule-like domains. *J Biol Chem* **273**, 3415-21.

Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J. L. (1990). Cyclin is a component of maturation-promoting factor from Xenopus. *Cell* **60**, 487-94.

Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J., and Schier, A. F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**, 75-9.

Goldman, D. S., Kiessling, A. A., and Cooper, G. M. (1988). Post-transcriptional processing suggests that c-mos functions as a maternal message in mouse eggs. *Oncogene* **3**, 159-62.

Gordo, A. C., Rodrigues, P., Kurokawa, M., Jellerette, T., Exley, G. E., Warner, C.,

and Fissore, R. (2002). Intracellular calcium oscillations signal apoptosis rather than activation in in vitro aged mouse eggs. *Biol Reprod* **66**, 1828-37.

Goud, P. T., Goud, A. P., Laverge, H., De Sutter, P., and Dhont, M. (1999). Effect of post-ovulatory age and calcium in the injection medium on the male pronucleus formation and metaphase entry following injection of human spermatozoa into golden hamster oocytes. *Mol Hum Reprod* **5**, 227-33.

Goudet, G., Mugnier, S., Callebaut, I., and Monget, P. (2008). Phylogenetic analysis and identification of pseudogenes reveal a progressive loss of zona pellucida genes during evolution of vertebrates. *Biol Reprod* **78**, 796-806.

Harper, E. H. (1904). The fertilisation and early development of the pigeon's egg. *American Journal of Anatomy* 3, 349-386.

He, C. L., Damiani, P., Ducibella, T., Takahashi, M., Tanzawa, K., Parys, J. B., and Fissore, R. A. (1999). Isoforms of the inositol 1,4,5-trisphosphate receptor are expressed in bovine oocytes and ovaries: the type-1 isoform is down-regulated by fertilization and by injection of adenophostin A. *Biol Reprod* **61**, 935-43.

Howes, L., and Jones, R. (2002). Interactions between zona pellucida

glycoproteins and sperm proacrosin/acrosin during fertilization. *J Reprod Immunol* **53**, 181-92.

Howlett, S. K., and Bolton, V. N. (1985). Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. *J Embryol Exp Morphol* **87**, 175-206.

Hrabia, A., Takagi, S., Ono, T., and Shimada, K. (2003). Fertilization and development of quail oocytes after intracytoplasmic sperm injection. *Biol Reprod* **69**, 1651-7.

Igarashi, H., Takahashi, E., Hiroi, M., and Doi, K. (1997). Aging-related changes in calcium oscillations in fertilized mouse oocytes. *Mol Reprod Dev* **48**, 383-90.

Jellerette, T., Kurokawa, M., Lee, B., Malcuit, C., Yoon, S. Y., Smyth, J., Vermassen, E., De Smedt, H., Parys, J. B., and Fissore, R. A. (2004). Cell cycle-coupled [Ca(2+)](i) oscillations in mouse zygotes and function of the inositol 1,4,5-trisphosphate receptor-1. *Dev Biol* **274**, 94-109.

Jones, K. T. (2004). Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization. *Mol Hum Reprod* **10**, 1-5.

Jones, K. T., Carroll, J., Merriman, J. A., Whittingham, D. G., and Kono, T. (1995). Repetitive sperm-induced Ca2+ transients in mouse oocytes are cell

cycle dependent. *Development* **121**, 3259-66.

Jones, K. T., Cruttwell, C., Parrington, J., and Swann, K. (1998). A mammalian sperm cytosolic phospholipase C activity generates inositol trisphosphate and causes Ca2+ release in sea urchin egg homogenates. *FEBS Lett* **437**, 297-300.

Kikuchi, K., Naito, K., Noguchi, J., Kaneko, H., and Tojo, H. (2002). Maturation/M-phase promoting factor regulates aging of porcine oocytes matured in vitro. *Cloning Stem Cells* **4**, 211-22.

Kikuchi, K., Naito, K., Noguchi, J., Shimada, A., Kaneko, H., Yamashita, M., Aoki, F., Tojo, H., and Toyoda, Y. (2000). Maturation/M-phase promoting factor: a regulator of aging in porcine oocytes. *Biol Reprod* **63**, 715-22.

Lawrence, Y., Whitaker, M., and Swann, K. (1997). Sperm-egg fusion is the prelude to the initial Ca2+ increase at fertilization in the mouse. *Development* **124**, 233-41.

Lee, B., Vermassen, E., Yoon, S. Y., Vanderheyden, V., Ito, J., Alfandari, D., De Smedt, H., Parys, J. B., and Fissore, R. A. (2006a). Phosphorylation of IP3R1 and the regulation of [Ca2+]i responses at fertilization: a role for the MAP kinase pathway. *Development* **133**, 4355-65.

Lee, B., Yoon, S. Y., and Fissore, R. A. (2006b). Regulation of fertilization-

initiated [Ca2+]i oscillations in mammalian eggs: a multi-pronged approach. *Semin Cell Dev Biol* **17**, 274-84.

Lee, J. H., and Campbell, K. H. (2008). Caffeine treatment prevents age-related changes in ovine oocytes and increases cell numbers in blastocysts produced by somatic cell nuclear transfer. *Cloning Stem Cells* **10**, 381-90.

Liang, C. G., Su, Y. Q., Fan, H. Y., Schatten, H., and Sun, Q. Y. (2007). Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. *Mol Endocrinol* **21**, 2037-55.

Ma, J., Zeng, F., Schultz, R. M., and Tseng, H. (2006). Basonuclin: a novel mammalian maternal-effect gene. *Development* **133**, 2053-62.

Ma, W., Zhang, D., Hou, Y., Li, Y. H., Sun, Q. Y., Sun, X. F., and Wang, W. H. (2005). Reduced expression of MAD2, BCL2, and MAP kinase activity in pig oocytes after in vitro aging are associated with defects in sister chromatid segregation during meiosis II and embryo fragmentation after activation. *Biol Reprod* **72**, 373-83.

Madgwick, S., and Jones, K. T. (2007). How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals Cytostatic Factor. *Cell Div* **2**, 4.

Maleszewski, M., Borsuk, E., Koziak, K., Maluchnik, D., and Tarkowski, A. K. (1999). Delayed sperm incorporation into parthenogenetic mouse eggs: sperm nucleus transformation and development of resulting embryos. *Mol Reprod Dev* **54**, 303-10.

Markoulaki, S., Matson, S., Abbott, A. L., and Ducibella, T. (2003). Oscillatory CaMKII activity in mouse egg activation. *Dev Biol* **258**, 464-74.

Masui, Y., and Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J Exp Zool* **177**, 129-45.

Mattioli, M., Galeati, G., Bacci, M. L., and Barboni, B. (1991). Changes in maturation-promoting activity in the cytoplasm of pig oocytes throughout maturation. *Mol Reprod Dev* **30**, 119-25.

Meyer, T., and Stryer, L. (1991). Calcium spiking. *Annu Rev Biophys Biophys Chem* **20**, 153-74.

Miao, Y. L., Kikuchi, K., Sun, Q. Y., and Schatten, H. (2009). Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Hum Reprod Update* **15**, 573-85.

Miller, D. J., Shi, X., and Burkin, H. (2002). Molecular basis of mammalian gamete binding. *Recent Prog Horm Res* **57**, 37-73.

Miyazaki, S., Shirakawa, H., Nakada, K., and Honda, Y. (1993). Essential role of the inositol 1,4,5-trisphosphate receptor/Ca2+ release channel in Ca2+ waves and Ca2+ oscillations at fertilization of mammalian eggs. *Dev Biol* **158**, 62-78.

Mizushima, S., Takagi, S., Ono, T., Atsumi, Y., Tsukada, A., Saito, N., Sasanami, T., Okabe, M., and Shimada, K. (2010). Novel method of gene transfer in birds: intracytoplasmic sperm injection for green fluorescent protein expression in quail blastoderms. *Biol Reprod* **83**, 965-9.

Mizushima, S., Takagi, S., Ono, T., Atsumi, Y., Tsukada, A., Saito, N., and Shimada, K. (2007). Possible role of calcium on oocyte development after intracytoplasmic sperm injection in quail (Coturnix japonica). *J Exp Zool A Ecol Genet Physiol* **307**, 647-53.

Mizushima, S., Takagi, S., Ono, T., Atsumi, Y., Tsukada, A., Saito, N., and Shimada, K. (2009). Phospholipase Czeta mRNA expression and its potency during spermatogenesis for activation of quail oocyte as a sperm factor. *Mol Reprod Dev* **76**, 1200-7.

Mongin, P., and Carter, N. W. (1977). Studies on the avian shell gland during egg formation: aqueous and electrolytic composition of the mucosa. *Br Poult Sci* **18**, 339-51.

Moran, E. T., Jr. (1987). Protein requirement, egg formation and the hen's

ovulatory cycle. *J Nutr* **117**, 612-8.

Morris, T. R. (1973). The effects of ahemeral light and dark cycles on egg production in the fowl. *Poult Sci* **52**, 423-45.

Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwabara, Y., and Miyazaki, S. (1997). Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. *Mol Hum Reprod* **3**, 1087-93.

Okamura, F., and Nishiyama, H. (1978). Penetration of spermatozoon into the ovum and transformation of the sperm nucleus into the male pronucleus in the domestic fowl, Gallus gallus. *Cell Tissue Res* **190**, 89-98.

Ono, T., Murakami, T., Mochii, M., Agata, K., Kino, K., Otsuka, K., Ohta, M., Mizutani, M., Yoshida, M., and Eguchi, G. (1994). A complete culture system for avian transgenesis, supporting quail embryos from the single-cell stage to hatching. *Dev Biol* **161**, 126-30.

Ono, T., Nakane, Y., Wadayama, T., Tsudzuki, M., Arisawa, K., Ninomiya, S., Suzuki, T., Mizutani, M., and Kagami, H. (2005). Culture system for embryos of blue-breasted quail from the blastoderm stage to hatching. *Exp Anim* **54**, 7-11.

Ozil, J. P. (1998). Role of calcium oscillations in mammalian egg activation:

experimental approach. *Biophys Chem* **72**, 141-52.

Ozil, J. P., Banrezes, B., Toth, S., Pan, H., and Schultz, R. M. (2006). Ca2+ oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Dev Biol* **300**, 534-44.

Palermo, G. D., Colombero, L. T., Schattman, G. L., Davis, O. K., and Rosenwaks, Z. (1996). Evolution of pregnancies and initial follow-up of newborns delivered after intracytoplasmic sperm injection. *JAMA* **276**, 1893-7.

Patterson, R. L., Boehning, D., and Snyder, S. H. (2004). Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annu Rev Biochem* **73**, 437-65.

Perry, M. M. (1987). Nuclear events from fertilisation to the early cleavage stages in the domestic fowl (Gallus domesticus). *J Anat* **150**, 99-109.

Pines, J., and Hunter, T. (1989). Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell* **58**, 833-46.

Rabbani, M. G., Sasanami, T., Mori, M., and Yoshizaki, N. (2006). Sperm-egg interaction is mediated by a sperm-associated body in quail. *Dev Growth Differ* **48**, 33-40.

Rabbani, M. G., Sasanami, T., Mori, M., and Yoshizaki, N. (2007). Characterization of the sperm-associated body and its role in the fertilization of the chicken Gallus domesticus. *Dev Growth Differ* **49**, 39-48.

Rime, H., Haccard, O., and Ozon, R. (1992). Activation of p34cdc2 kinase by cyclin is negatively regulated by cyclic amp-dependent protein kinase in Xenopus oocytes. *Dev Biol* **151**, 105-10.

Sagata, N., Daar, I., Oskarsson, M., Showalter, S. D., and Vande Woude, G. F. (1989). The product of the mos proto-oncogene as a candidate "initiator" for oocyte maturation. *Science* **245**, 643-6.

Sakai, N., and Endo, A. (1988). Effects of delayed mating on preimplantation embryos in spontaneously ovulated mice. *Gamete Res* **19**, 381-5.

Sakurai, A., Oda, S., Kuwabara, Y., and Miyazaki, S. (1999). Fertilization, embryonic development, and offspring from mouse eggs injected with round spermatids combined with Ca2+ oscillation-inducing sperm factor. *Mol Hum Reprod* **5**, 132-8.

Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K., and Lai, F. A. (2002). PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* **129**, 3533-44.

Schultz, R. M., and Kopf, G. S. (1995). Molecular basis of mammalian egg activation. *Curr Top Dev Biol* **30**, 21-62.

Snell, W. J., and White, J. M. (1996). The molecules of mammalian fertilization. *Cell* **85**, 629-37.

Stepinska, U., and Olszanska, B. (2003). DNase I and II present in avian oocytes: a possible involvement in sperm degradation at polyspermic fertilisation. *Zygote* **11**, 35-42.

Stevens, L. (1991). Egg white proteins. *Comp Biochem Physiol B* **100**, 1-9.

Stricker, S. A. (1996). Repetitive calcium waves induced by fertilization in the nemertean worm Cerebratulus lacteus. *Dev Biol* **176**, 243-63.

Stricker, S. A. (1997). Intracellular injections of a soluble sperm factor trigger calcium oscillations and meiotic maturation in unfertilized oocytes of a marine worm. *Dev Biol* **186**, 185-201.

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol* **211**, 157-76.

Sultana, F., Mao, K. M., and Yoshizaki, N. (2004). Possible involvement of a sperm-associated body in the process of fertilization in quail. *Zoolog*

Sci **21**, 851-8.

Swann, K. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development* **110**, 1295-302.

Swann, K. (1992). Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store. *Biochem J* **287 (Pt 1)**, 79-84.

Takagi, S., Ono, T., Tsukada, A., Atsumi, Y., Mizushima, S., Saito, N., and Shimada, K. (2007). Fertilization and blastoderm development of quail oocytes after intracytoplasmic injection of chicken sperm bearing the W chromosome. *Poult Sci* **86**, 937-43.

Takahashi, T., Saito, H., Hiroi, M., Doi, K., and Takahashi, E. (2000). Effects of aging on inositol 1,4,5-triphosphate-induced Ca(2+) release in unfertilized mouse oocytes. *Mol Reprod Dev* **55**, 299-306.

Takeuchi, Y., Nishimura, K., Aoki, N., Adachi, T., Sato, C., Kitajima, K., and Matsuda, T. (1999). A 42-kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC family glycoproteins in mammalian Zona pellucida. Its first identification, cDNA cloning and granulosa cell-specific expression. *Eur J Biochem* **260**, 736-42.

Tam, O. H., Aravin, A. A., Stein, P., Girard, A., Murchison, E. P., Cheloufi, S., Hodges, E., Anger, M., Sachidanandam, R., Schultz, R. M., and Hannon, G. J. (2008). Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* **453**, 534-8.

Tang, F., Kaneda, M., O'Carroll, D., Hajkova, P., Barton, S. C., Sun, Y. A., Lee, C., Tarakhovsky, A., Lao, K., and Surani, M. A. (2007). Maternal microRNAs are essential for mouse zygotic development. *Genes Dev* **21**, 644-8.

Tarin, J. J., Perez-Albala, S., Aguilar, A., Minarro, J., Hermenegildo, C., and Cano, A. (1999). Long-term effects of postovulatory aging of mouse oocytes on offspring: a two-generational study. *Biol Reprod* **61**, 1347-55.

Tatone, C., Carbone, M. C., Gallo, R., Delle Monache, S., Di Cola, M., Alesse, E., and Amicarelli, F. (2006). Age-associated changes in mouse oocytes during postovulatory in vitro culture: possible role for meiotic kinases and survival factor BCL2. *Biol Reprod* **74**, 395-402.

Tian, X. C., Lonergan, P., Jeong, B. S., Evans, A. C., and Yang, X. (2002). Association of MPF, MAPK, and nuclear progression dynamics during activation of young and aged bovine oocytes. *Mol Reprod Dev* **62**, 132-8.

Tong, Z. B., Gold, L., Pfeifer, K. E., Dorward, H., Lee, E., Bondy, C. A., Dean, J., and Nelson, L. M. (2000). Mater, a maternal effect gene required for

early embryonic development in mice. *Nat Genet* **26**, 267-8.

Tripathi, A., Kumar, K. V., and Chaube, S. K. (2010). Meiotic cell cycle arrest in mammalian oocytes. *J Cell Physiol* **223**, 592-600.

Tsai, J. Y., and Silver, L. M. (1996). Sperm-egg binding protein or proto-oncogene? *Science* **271**, 1432-4; author reply 1434-5.

Tulsiani, D. R., and Abou-Haila, A. (2011). Molecular events that regulate mammalian fertilization. *Minerva Ginecol* **63**, 103-18.

Tunquist, B. J., and Maller, J. L. (2003). Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes Dev* **17**, 683-710.

Verbert, L., Lee, B., Kocks, S. L., Assefa, Z., Parys, J. B., Missiaen, L., Callewaert, G., Fissore, R. A., De Smedt, H., and Bultynck, G. (2008). Caspase-3-truncated type 1 inositol 1,4,5-trisphosphate receptor enhances intracellular Ca2+ leak and disturbs Ca2+ signalling. *Biol Cell* **100**, 39-49.

Waddington, D., Gribbin, C., Sterling, R. J., Sang, H. M., and Perry, M. M. (1998). Chronology of events in the first cell cycle of the polyspermic egg of the domestic fowl (Gallus domesticus). *Int J Dev Biol* **42**, 625-8.

Wang, Q., and Sun, Q. Y. (2007). Evaluation of oocyte quality: morphological,

cellular and molecular predictors. *Reprod Fertil Dev* **19**, 1-12.

Wang, Q. T., Piotrowska, K., Ciemerych, M. A., Milenkovic, L., Scott, M. P., Davis, R. W., and Zernicka-Goetz, M. (2004). A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. *Dev Cell* **6**, 133-44.

Wang, X. (2001). The expanding role of mitochondria in apoptosis. *Genes Dev* **15**, 2922-33.

Ward, C. R., and Kopf, G. S. (1993). Molecular events mediating sperm activation. *Dev Biol* **158**, 9-34.

Wassarman, P. M. (1988). Zona pellucida glycoproteins. *Annu Rev Biochem* **57**, 415-42.

Wassarman, P. M., Jovine, L., and Litscher, E. S. (2001). A profile of fertilization in mammals. *Nat Cell Biol* **3**, E59-64.

Watanabe, T., Totoki, Y., Toyoda, A., Kaneda, M., Kuramochi-Miyagawa, S., Obata, Y., Chiba, H., Kohara, Y., Kono, T., Nakano, T., Surani, M. A., Sakaki, Y., and Sasaki, H. (2008). Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature* **453**, 539-43.

Wilding, M., Dale, B., Marino, M., di Matteo, L., Alviggi, C., Pisaturo, M. L., Lombardi, L., and De Placido, G. (2001). Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod* **16**, 909-17.

Woolley, D. M., and Bozkurt, H. H. (1995). The distal sperm flagellum: its potential for motility after separation from the basal structures. *J Exp Biol* **198**, 1469-81.

Wramsby, H., and Hansson, A. (1984). Technique to examine human sperm chromosomes using zona-free hamster eggs. *Arch Androl* **12**, 79-83.

Wu, H., He, C. L., and Fissore, R. A. (1997). Injection of a porcine sperm factor triggers calcium oscillations in mouse oocytes and bovine eggs. *Mol Reprod Dev* **46**, 176-89.

Yanagimachi, R. (1994). Fertility of mammalian spermatozoa: its development and relativity. *Zygote* **2**, 371-2.

SUMMARY IN KOREAN

조류의 자연적인 수정은 난자가 배란 후 수란관의 첫 부분인 누두부(infundibulum)에 위치하였을 때만 이루어진다고 알려져 있다. 난자가 누두부를 통과한 후 난백분비부 (magnum)에 들어가면 난백 분비물로 둘러싸이게 되어 정자가 난자의 표면에 결합하는 것을 방해한다. 하지만 난세포질내 정자 주입법 (Intracytoplasmic sperm injection)을 이용하면 난백분비부에서 채취한 난자의 분비물을 직접 제거하고 정자를 주입하기 때문에 수정을 성공적을 할 수 있다는 지난 연구들이 보고 되었다. 하지만 난자를 수란관의 누두부나 난백분비부에서 채취하기 위해서는 메추리를 희생할 수밖에 없었다. 난자가 난각이 형성되는 부분인 관상난각선 (shell gland)에 위치하면 복부 마사지를 통하여 난자를 채취할 수 있다. 관상난각선에서 채취한 난자가 성공적으로 수정이 된다면 앞으로의 난세포질내 정자 주입법을 이용한 연구에 메추리를 희생하지 않아도 되며 멸종위기조류를 보호하는 연구에 적용될 수 있다. 따라서 본 연구의 목적은 메추리 난자의 배란 후 발생 능력과 분자적 특성을 알아보는데 있다.

메추리의 난자를 배란이 일어나고 (1) 0.5-1.5 (2) 3.5-4 (3) 5-6 (4) 9-10 (5) 22-23 시간 뒤에 수란관에서 채취하여 난세포질내 정자 주입법을 통하여 메추리 정자와 inositol 1,4,5-trisphosphate (IP_3)를 주입하여 체외의 수정을 유도하였다. 주입된 난자를 DMEM에 24 시간 배양한 후 배반엽 (blastoderm)의 형태의 관찰과 4.6-diamidino-2-phenylindole (DAPI) 염색을 통하여 수정 여부를 확인하였다. 배란 후 0.5-1.5 시간 후에 채취된 난자는 60%의 가장 높은 수정률을 보였고 더 늦은 시간에 채취한 난자들에서는 수정률이 36.4-18.8%로 낮아지는 것을 확인할 수 있었다. 또한 배란 후 0.5-1.5 시간 후 채취한 난

자들의 16.7%가 Eyal-Giladi and Kochav (EG&K) stage IV 이상의 발달한 것을 관찰할 수 있었다. 하지만 더 늦을 시간에 채취한 난자들은 성공적으로 수정이 되었어도 EG&K stage IV 전에 배아 발달이 중지되는 것을 확인하였다. 본 연구 결과는 메추리 난자의 수정 능력이 산란이 되는 시기까지 유지된다는 것을 시사한다.

` 메추리 난자의 수정률과 배아 발달을 개선하기 위해서는 난자의 배아 수정과 발달에 관련된 메커니즘에 대한 연구가 필요하다. 따라서 본 연구는 난자의 분자적 특성이 배란 후 시간이 지남에 따라 어떻게 변하는지를 관찰하기 mRNA 발현 변화를 분석하였다. 배란 후 (1) 0.5-1.5, (2) 5-6, (3) 22-23 시간 뒤에 채취된 난자에 난세포질내 정자 주입법을 이용하여 IP_3를 주입한 후 DMEM에 3 시간 배양한 후 각 난자의 배반엽에서 RNA를 분리하여 cDNA를 합성 후 양적중합효소 연쇄반응을 수행하였다. 실험결과 IP_3 수용체의 유전자인 *ITPR1* 과 *ITPR2* 의 mRNA 발현량이 배란 후 0.5-1.5 시간 뒤에 채취된 난자에서 가장 높았으나 배란 후 5-6시간 그리고 22-23 시간 뒤에 채취한 난자에서는 발현량이 낮아지는 것을 관찰하였다. 본 연구 결과는 IP_3 수용체 타입 1 과 타입 2 가 IP_3로 유도된 칼슘이 난자세포질로 방출되는 과정이나 난모세포 자극 (oocyte activation)에 관련된 있음을 시사한다. 관상난각선과 산란된 난자에서 관찰된 낮은 *ITPR1* 과 *ITPR2* mRNA의 발현량은 낮은 수정률과 배아 발달에도 관련이 있음을 시사한다.

본 연구는 조류 난자의 수정과 발달 능력을 이해하고, 탐구하는데 도움이 될 뿐만 아니라 멸종위기조류를 보호하고 동물을 희생하지 않고 난세포질내 정자 주입법을 이용한 연구를 진행하는데 기여를 할 것이라 사료된다.

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