METHOD FOR ISOLATING IMMATURE CHICKEN OOCYTES

by

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Abstract

Knowledge of the development of the avian oocyte has been difficult to obtain due to the physical package of the oocyte at ovulation. The ability to isolate and culture avian oocytes, prior to the accumulation of large amounts of yolk and packing in albumen and shell, would allow the study of oocyte development. The objective of the described studies was to develop methods for dispersing and isolating the oocytes of immature chickens. Three concentrations of proteolytic enzymes were tested for efficiency for disaggregating ovarian tissue. Three methods were testing for removing contaminating erythrocytes (RBCs) and fibroblasts from the oocyte preparations. These isolation methods included: lysing of red blood cells and attachment of fibroblasts to a culture surface, Percoll density gradient centrifugation, and depletion of contaminating fibroblasts by binding to specific anti-fibroblast antibodies and lysing red blood cells. Increasing the concentration of Collagenase Type II in the enzyme mixture led to the release of a larger number of oocytes. While the lysing of red blood cells removed the red blood cells very effectively, depleting fibroblasts through attachment to the cell culture surface to allow decantation of the oocytes was not an effective method of disposing of the fibroblasts. Percoll density gradient centrifugation was effective in removing red blood cells from the oocytes. However, the remaining oocytes appeared to be damaged, and non-lysed fibroblasts remained in the solution. Antibody binding was a very effective method of removing fibroblasts from the cell solution, and the lysing of red

blood cells in addition proved to be ideal for eliminating contaminating cell types. This experiment concluded that it is feasible to disperse the ovarian tissue in immature chicks and then isolate their oocytes for study.

To analyze the immature oocytes further, flow cytometry was utilized to measure the DNA content of the cells. This enabled the assessment of the oocytes' current position in the cell cycle. It was found that oocytes in the three-week-old chick have not yet undergone meiosis and are diploid. Studies focused on the cell cycle positioning of the immature oocyte could lead to significant advancements in reproductive efficiencies, and control of the offspring.

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I. INTRODUCTION

The megalecithal nature of the avian oocyte (egg cell) makes it particularly difficult to manipulate for study. As the oocyte travels through the oviduct, the shell, albumen, and yolk are secreted upon it, making the oocyte difficult to access for purposes of cell culture. Thus, studies focusing on avian oocyte development have utilized preserved histological sections of the ovary containing the embedded oocytes. While this method of study is useful, it does not allow for the manipulation of the live immature avian oocyte. A comprehensive method for isolating and culturing live avian oocytes is needed to advance the study of oocyte development.

II. LITERATURE REVIEW

AVIAN OOCYTE ORIGIN

The embryonic ovary of the chick embryo contains up to 680,000 oocytes prior to hatching. Within a few days after hatching this number drops to 480,000 (Hughes, 1963). The events that occur to produce these oocytes are fairly well characterized up to the point that the primordial germ cells take up residence in the embryonic ovary. The remaining oocytes, at sexual maturation of the hen, develop into the familiar yolky follicles that are then ovulated to produce a chicken egg. The differentiation and reduction in the number of the oocytes subsequent to embedding in the ovary are less well known. Also unknown is the exact moment at which cell cycle events, particularly meiotic events that determine the sex of the gamete, occur.

Primordial Germ Cells

Primordial germ cells (PGCs) are direct predecessors to germ cells, which are the precursors for the spermatozoa and the oocyte (Gilbert, 2003). In the chicken, primordial germ cells originate in the epiblast of the blastocoel, far from where they will eventually develop (Eyal-Giladi et al., 1981). The developing PGCs are associated with the central disc of the area pellucida, where they differentiate (Ginsburg & Eyal-Giladi, 1987; Nakamura, 2007). As the embryo progresses, the PGCs will slowly migrate from the

epiblast into the hypoblast (Karagenc, 1996). Here, they will travel anteriorly, along with the hypoblast cells, toward the germinal crescent (Clawson, 1969). PGCs were first observed in the germinal crescent by Swift in 1914.

Once the circulatory system in the germinal crescent is formed, the PGCs will migrate into the developing blood vessels (Fujimoto, 1976b). The circulatory system will fill with blood, and carry the primordial germ cells to the germinal ridge (Fijimoto, 1976a.). Once the PGCs are circulated close to the germinal ridge, they actively migrate from the circulatory system to the germinal ridge (Ando and Fujimoto, 1983). Most of the PGCs should be situated in the germinal ridge on the third day of incubation (Fujimoto, 1976). While the mode of this active migration is not completely agreed upon, there are several factors that could influence the primordial germ cells' active migration into the germinal ridge, and the future site of the gonads. These factors include: chemotaxic factors (Dubois and Croisille, 1970; Godin, 1990), active, independent movement using pseudopodial processes (Meyer, 1964), physical contact guidance (Kuwana, 1984), and extracellular matrices (Urven, 1989). Nakamura et al. (2007) found that primordial germ cells in the chick proliferate throughout the early stages of development.

Primordial germ cells are easily distinguishable from the surrounding tissue due to their unique appearance. The avian PGCs' distinctive characteristics include its larger size (Swift, 1914; Goldsmith, 1928), large, eccentric nucleus, spherical shape (Abdel-Malek, 1950), appearance of lipid droplets in the cytoplasm (Kuwana, 1993), unique chromatin arrangement, and the presence of yolk material (Goldsmith, 1928). Since primordial germ cells have higher glycoprotein content than the surrounding cells, they

may be successfully distinguished using periodic acid Schiff staining (Meyer, 1960). The immunohistochemical marker, stage-specific embryonic antigen-1 (SSEA-1), has been found to be an effective identifier of primordial germ cells (Hamburger and Hamilton, 1951). A monoclonal antibody against mouse embryonal carcinoma cells (EMA-1) can also serve as a marker for PGCs to detect germ cells beneath the epiblast when they first differentiate, migrate, and colonize in the germinal crescent (Urven et al., 1988). While these molecular markers have been effective in detecting chicken primordial germ cells, the most effective molecular marker for primordial germ cells in the chicken is anti-chicken vasa homolog (CVH) antibody, which will detect CVH proteins when used in western blot analysis (Tsunekawa, 2000). PGCs can even be cryopreserved using liquid nitrogen (Moore et al., 2006).

Primordial germ cells in mammals are very similar to those of avians in appearance (Spiegelman and Bennett, 1973). The posterior area of the epiblast houses the emerging primordial germ cells (Gilbert, 2003). At about day 6.5 of development, cells in the ectoderm secrete bone morphogenic protein 4 (BMP4). This encourages the development of primordial germ cells in the surrounding cells, and it has been found that in the absence of BMP4 expression no primordial germ cells develop (Lawson et al., 1999). PGCs in mammals migrate out of the posterior primitive streak region of the epiblast and into the endoderm in the developing hindgut (Anderson, 2000; Molyneaux, 2001). The primordial germ cells will eventually spread out through the entire length of the hind gut (McLaren, 2003). They then leave the hindgut, travel through the embryo, and eventually into the developing gonads (Anderson, 2000; Molyneaux, 2001). The primordial germ cells will have arrived in the gonads by day 11.5 of development. At

this time they have grown in number to a population of 2,500-5,000 primordial germ cells (Gilbert, 2003).

The study of primordial germ cells in the mouse was revolutionized when it was found that these cells contain a relatively high level of tissue nonspecific alkaline phosphatase (TNAP) activity. By using TNAP as a marker, scientists in the 1950s were able to track the migratory pathway of the mammalian primordial germ cells from day eight of development (McLaren, 2003). It has been shown that β1 integrins are actually required for mouse primordial germ cells to actively travel to the developing gonads (Anderson, 1999). However, studies have revealed that primordial germ cells in mammals have the ability to move under their own devices using amoeboidal movement until they enter into the developing gonads (Donovan et al., 1986). The germ cells form a network as they move toward the developing gonads by touching one another with extending processes (Gompers et al., 1994). Godin et al. (1990) discovered that there are intrinsic factors released from the developing gonads that influence the direction of primordial germ cell migration. Glycoproteins in the extracellular matrix, such as fibronectin, could play a major role in influencing primordial germ cell migration. A moderate level of the extracellular matrix glycoprotein, fibronectin, is thought to be ideal for the process of migration. Just before migration occurs fibronectin production is drastically reduced. This could be to signal the timing of migration and to allow the cells to move more freely, but also have enough fibronectin to adhere to cells to aid in migration. It has also been suggested that fibronectin may be involved in guiding the primordial germ cells into the developing gonads (French-Constant et al., 1991). It has been shown that the cells lining the migration pathway the primordial germ cells travel

along into the developing gonads actively produce stem cell factor. This compound is essential for primordial germ cells to proliferate in the embryo, as well as in vitro (Pesce et al., 1993).

OOCYTE DEVELOPMENT

Cell Cycle of Gametes

Cellular reproduction is essential to life, and enables organisms to replace dying cells or repair damage to tissue. The cell cycle is divided into several distinct steps. Mitosis is the stage of the cell cycle in which the cell actually replicates itself in several steps. These steps are highly regulated to ensure they are carried out correctly and completely. The first occurrence in cellular reproduction is to copy the cell's content, and the second is to divide those copies into two distinct daughter cells, which are equipped with all of the contents the original cell held.

In addition to mitosis, primordial germ cells must also undergo meiosis, which will transform them into either oocytes or spermatocytes. This transformation is accomplished through one round of chromosome replication, but two nuclear divisions, which result in a decrease in chromosome number to a haploid state. Diploid cells contain two slightly different copies of the same chromosome, each inherited by one parent. Haploid gametes join together to create a diploid individual with the same number of chromosomes as the parent organism. Meiosis must also be closely regulated by the cell to avoid a mistake in the number of chromosomes passed along to the offspring (Morgan, 2007).

Mitosis

During development and differention of cells, they go through a number of processes commonly referred to as the "cell cycle". Somatic cells undergo a mitotic cell cycle, while sex cells go through both mitotic and meiotic cycles.

The two main stages of the mitotic cycle are: Interphase and the M (mitotic) phase. These phases are further subdivided based on the intracellular events that occur. Interphase is sometimes referred to as the resting phase. However, it is not just a resting phase of the cell. It has a number of sub-phases, and plays an important role in the preparation for mitosis. One of the phases in interphase is the S phase, or the synthesis phase, and it results in a complete additional copy of DNA. Chromosome duplication during the S phase results in two sister chromatids. Duplication begins at specific points along the chromosome called replication origins. Proteins will unwind the DNA double helix in both directions and allow enzymes to synthesize DNA. When DNA replication is complete specialized proteins called histones will wind the DNA into chromosomes. Centrosomes, which organize microtubules, are also duplicated during the S phase (Morgan, 2007).

The M phase, or mitosis phase, consists of two components: mitosis, or nuclear division, and cytokinesis, the division of the cytoplasm. During the metaphase portion of M phase, microtubules form the mitotic spindle. This structure plays the key role in pulling the chromatids apart. Cytokinesis will then divide the organelle into two distinct cells and separate the cytoplasm (Morgan, 2007).

The S phase and M phase are separated by gap phases (G1 and G2). The gap phases serve to clearly divide the S and M phases, and also allow a time specifically for cell growth. Cell growth and cell division are regulated completely independently. A cell can undergo growth without division and visa versa (Morgan, 2007).

Mitosis is divided into four basic steps including: prophase, metaphase, anaphase, and telophase (Berrill, 1971). During the first phase, prophase, the chromosomes within the nucleus condense together to prepare for mitosis. At this point, the mitotic cells can be identified using light microscopy (Morgan, 2007). The nucleolus and the nuclear membrane of the cell disappear (Berrill, 1971). Metaphase is characterized by the appearance of the spindle fibers that join the chromosomes to the centrioles. During metaphase, the chromatids are aligned in the center of the cell. Anaphase is the most stunning part of mitosis because the chromosomes dramatically move toward opposite poles of the cell (Morgan, 2007). Telophase is characterized by the reappearance of the nucleolus and the nuclear membrane (Berrill, 1971). One centrosome remains with each new daughter cell, but the mitotic spindle is taken down (Morgan, 2007).

Mitosis in germ cells is unique to somatic cells verses the germ cells because the G1 phase is practically nonexistent in germ cells. The regulatory mechanisms that take place in somatic cells to ensure the cell is given a proper amount of time to recover from cytokinesis and mitosis and grow are not present in the germ cell cycle. Mitosis in germ cells is also unique because the cells form cysts. The cysts grow through mitosis, and the cells are connected by bridges that allow certain substances to pass from cell to cell (Pepling and Spradling, 1998).

Control of Cell Cycle

External signals usually dictate whether or not a cell will progress through the cell cycle, but there are also three regulatory checkpoints that control progression during the cell cycle: Start (G1/S), Mitosis (G2/M), and Metaphase-to-Anaphase. The G1/S, or Start, checkpoint serves to promote DNA synthesis. Next, the G2/M checkpoint serves to promote spindle activity. Lastly, the Metaphase-to-Anaphase transition checkpoint serves to ensure sister chromatid separation and completion of mitosis and cytokinesis. Each of these checkpoints has multiple control mechanisms to ensure proper progression through the cell cycle. The cell cycle control system is adaptable and robust. It consists of cyclin-Cdk switches as well as feedback loops. These control mechanisms have an all-or-nothing effect to ensure the completion of steps during mitosis (Pavletich, 1999; Morgan, 2007).

The cell cycle is mostly controlled by phosphorylation, which will change a substrate's interaction with other proteins. The phosphates used here are derived from adenosine triphosphate (ATP). Cyclin-dependent kinases (Cdks) catalyze the covalent bonding of these phosphate groups to their protein substrates, and subsequently activate certain substrates at certain times to control the cell cycle. There may be hundreds of Cdk substrates (Ubersax et al., 2003). These cyclin-dependent kinases are in a family of serine/threonine protein kinase enzymes (Jeffrey et al., 1995). This family of enzymes is composed of very small proteins, which are between 34 and 40 kDa (Morgan, 2007). To completely activate a Cdk, the phosphorylation of a threonine residue on the Cdk is needed in addition to the binding of a cyclin. This phosphorylation is catalyzed by Cdk-activating kinase (CAK), which is a Cdk that will phosphorylate other Cdks. CAK

activity remains constant and high throughout the cell cycle (Jeffrey et al., 1995; Pavletich, 1999; Morgan, 2007). Cell division control protein 2 (also called Cdc28) and Cdk2 are the Cdks of the cell cycle (Morgan, 1997). The binding of cyclins to the Cdks will activate the Cdk by changing its structure (Jeffrey, 1995).

While these Cdks help push the cell through the cell cycle, there is also a molecular check system to inhibit the progression of the cell through the cycle (Morgan, 2007). Cdk is inhibited by the additional phosphorylation of Thr14, a threonine residue, and Tyr 15, a tyrosine residue. Wee1 is an enzyme phosphorylating Tyr 15. Myt1 is an enzyme that phosphorylates Thr14 and Tyr15. Cdc25 is an enzyme that dephosphorylates both Thr14 and Tyr15. These enzymes allow for the regulation of the cyclin-Cdk complex (Morgan, 2007).

Cyclins are proteins that aid in regulating the cell cycle by binding and activating cyclin-dependent kinases (Morgan, 1997). Both cyclins and cyclin-dependent kinases are involved in other cellular process, but certain ones are involved in regulating the cell cycle as well. Cyclins can guide the Cdks to cellular structures or substrates within the cell. This can help push the cell cycle along. The concentration of the cyclin-dependent kinases does not change over the progression of the cell cycle, rather the concentrations of the cyclins are regulated to cause an increase or decrease in Cdk activity (Morgan, 1997; Ubersax, 2003). The cell cycle control system controls concentrations of cyclins by down regulating their gene expression and increasing proteolysis of cyclins (Morgan, 1997). There are four classes of cyclins involved in cell cycle control. These classes include: G1/S, S, M, and G1 (Morgan, 2007).

G1/S cyclin-Cdk complex activates the progression of the cell through the Start checkpoint, and allows DNA replication to begin by increasing in concentration during late G1 phase. The concentration of G1/S cyclins (Cyclin E) will then decrease during early S phase. The concentration of S cyclins will begin to increase in the late G1 phase. S cyclins (Cyclin A) bind with Cdks to stimulate DNA replication, and their concentrations remain high through S, G2, and early M phases (Morgan, 2007).

The M cyclins (Cyclin B) increase in concentration at the beginning of mitosis and peak at metaphase. M cyclin-Cdk complexes stimulate the development of the mitotic spindle and the alignment of the sister chromatids (Morgan, 2007). mRNAs encoding M cyclins are housed in the cytoplasm of the cell (Gilbert, 2003).

The G1 cyclins (Cyclin D) promote cell growth. Their concentration levels do not rise and fall, but increase steadily throughout the cell cycle (Morgan, 2007). During G1, there are three basic regulatory mechanisms happening: there is an increase in the concentration of Cdk inhibitors, anaphase-promoting complex is active, and the expression of cyclin genes is suppressed by inhibitory gene regulatory proteins (Morgan, 2007). Cdk inhibitor proteins (CKIs) will inhibit the Cdks by binding to the Cdks and inactivating them. A protein called anaphase-promoting complex (APC) assures the cell has an adequate gap phase by degrading S and M phase cyclins, but leaving the G1/S cyclins. The genes encoding for S and M phase cyclins are also suppressed, and there is an increase in the concentration of Cdk inhibitors. These three regulatory mechanisms are important to maintain the cell in the G1 phase (Morgan, 2007).

To push the cell into the S phase, there is an increase in G1/S and S cyclin gene expression, and an activation of G1/S cyclin-dependent kinase. To activate chromosome

duplication, the S Cdks will phosphorylate proteins associated with DNA replication. Once the cell is involved in the S phase, the G1/S Cdks will signal the destruction of G1/S cyclins and the reduction of G1/S cyclin gene expression (Morgan, 2007).

To enter into the M phase, there is an increase in M-cyclin gene expression and, therefore, an increase in the concentration of M cyclins. The concentration of M cyclin-Cdk complexes increases during G2, and when the cell enters the mitotic phase these complexes are activated. To stimulate the metaphase-to-anaphase transition, the M-Cdks will stimulate the activation of APC. These APCs will stimulate enzymes that destroy the proteins that connect the sister chromatids. APC destroys cyclins, which causes dephosphorylation of Cdk substrates and allows for spindle disassembly. During the later part of the M phase, there is a decrease in S and M cyclin gene expression, and the APCs will destroy S and M cyclins. There is an increase in Cdk inhibitor proteins during late M phase as well (Morgan, 2007).

Cyclin-dependent kinases are inhibited by Cip or INK4 subunits (Pavletich, 1999). To inhibit Cdks, Cip will bind to the Cdk-cyclin complex. This is accomplished by the bound Cip physically blocking a critical ATP molecule binding site. The Cip inhibitor also changes the shape of the cyclin binding site on the Cdk, preventing a cyclin from binding.

The INK4 will inhibit Cdks through more indirect means. This subunit will bind to the lone Cdk to block the binding of cyclins. When the INK4 binds to the Cdk, it changes the structure of the cyclin binding site, thus indirectly blocking the binding of the cyclin. INK4 binding also distorts an adjacent ATP binding site, which inhibits ATP

binding and interferes with any bound ATP. INK4 is specifically for G1 phase Cdks, but Cips are not limited to one phase (Pavletich, 1999; Morgan, 2007).

Methods for Studying/Identifying Position

The cell cycle can more easily be studied in vitro. This can be accomplished by harvesting cells from the species of interest and then immortalizing those cells in an established cell line. Transformed cell lines should not be used to analyze cell cycle regulation because the regulatory mechanisms associated with these cell lines are altered. The study of cell cycle control has been studied much more extensively in mammals as opposed to avians. Genetic analysis in mammals is still quite difficult, but there are a few methods that have been somewhat successful (Morgan, 2007).

To study gene expression affecting cell-cycle control, altering protein and gene function is ideal. Short interfering RNAs (siRNAs) are used as a type of RNA interference to silence specific genes. They are small RNA fragments that contain a portion of the target RNA sequence and can be introduced into the cell. This double strand targets the RNA strand of interest. Enzymes in the cell destroy the target RNA, reducing the concentration of the target RNA and thus the protein of interest (Novina and Sharp, 2004).

Another method of studying gene expression in mammals is to cause specific gene disruption by using DNA vectors to homologously recombine and “knock out” the target gene. This technique is often used in embryonic stem cells, which are then used to produce animals with the knock out. This specific gene disruption technique can also be

used to study cells in primary cell cultures, usually utilizing embryonic fibroblasts from transgenic embryos (Morgan, 2007).

The position of the cell in the cell cycle can be observed through simple light microscopy. While this can be more difficult in higher organisms than in yeast, the cells will unattached themselves and have a distinct round appearance during the M phase. Crystallographic studies can be used to study the structural aspects of enzymes and substrates involved in the regulation of the cell cycle (Pavletich, 1999). Fluorescent labeling of chromosomes or the mitotic spindle is a more common approach to assessing the cell cycle stage of cells. There are several methods of applying fluorescent labeling to cell cycle analysis. The first is to fix cells onto a microscope slide and apply a fluorescent dye that will clearly mark the chromosomes or the mitotic spindle of the cell. The second method of applying fluorescent labeling to cell cycle analysis is to apply an antibody, which will bind to a specific structure within the cell and then attach a second, fluorescent antibody. A third method is to label protein structures in live cells. This can be accomplished by connecting a gene from jellyfish, which will encode for green fluorescent protein (GFP) to a target gene within the live cells. This will result in a fluorescent fusion protein that can label the target structure within the cell (Morgan, 2007).

Another method for studying the position of cells within the cell cycle is flow cytometry. Cells treated with a fluorescent DNA dye run through a flow cytometer, which will assess the DNA content through the amount of fluorescence. An example of this technique is bromodeoxyuridine (BrdU), which can be used to label cells in the S

phase. The flow cytometer is used to detect the amount of cells in the S phase, replicating DNA (Morgan, 2007).

Meiosis

The goal of meiotic divisions, or maturation divisions, is to reduce the number of chromosomes in the gamete to half the species number, or to the haploid condition. This will be accomplished by the cell only undergoing DNA replication once, but cellular division twice (Bishop, 2004; Patten, 1971). During oogenesis, the point is also to produce a cell that is equipped to maintain itself and support a developing zygote if fertilization is to occur. This means that the cell must contain not only the genetic material needed to pass down to potential offspring, but also all of the organelles, metabolic substrates, and cytoplasmic enzymes that the oocyte itself and a potential embryo would need to sustain itself (Gilbert, 2003).

Just before meiosis begins the gamete undergoes a time of DNA synthesis resulting with the cell ending up with four copies of each chromosome. This phase is called the S phase. The S phase in meiosis is much longer than the S phase in mitosis (Morgan, 176-179). The chromosomes will appear as two chromatids joined at a centromere. The nuclei of germ cells in the female chick embryo present a different structure during the premeiotic synthesis phase, or S phase, than during the premitotic S phase (Callebaut, 1967).

Although the chromatin of the interphase nucleus appears to be jumbled mess, it is now believed that it is orderly chaos. The nuclear membrane is thought to play a big part in organizing the chromatin for DNA synthesis. The nuclear envelope will bring the

chromosomes together, and the DNA synthesis may be initiated at the point of attachment to the envelope (Comings, 1967).

The germ cell will then enter prophase, where the objective is to breakdown the nuclear envelope and align the homologous chromosomes (Gilbert, 2003). There are five stages of prophase in meiosis. The first phase, called leptotene, is characterized by the chromatin being stretched out thinly. The zygotene phase is the second phase of prophase, where synapsis occurs. Synapsis is defined by the homologous chromosomes pairing up. The nuclear membrane must be present for this to occur (Gilbert, 2003). Comings (1967) has suggested that it is the nuclear envelope that brings the chromosomes together. The third phase of prophase is the pachytene phase. Crossing over is possible during pachytene phase. During this phase, the chromosomes thicken and shorten. The forth phase of prophase is the diplotene phase. Crossing over can still occur during the diplotene phase, increasing the chance for genetic diversity. The two chromosomes will start to separate at this point, but will remain connected at interspaced lengths along the chromatin called chiasmata. Transcription is occurring at this point (Gilbert, 2003). The chromosomes are called "lampbrush" chromosomes because of the appearance of the chromosomes as they produce RNA (Hill and MacGregor, 1980). The final, fifth stage of prophase is the diakinesis phase. During this phase the kinetochores will split up and the chromosomes will only be joined at the tips. The nuclear membrane will be broken down so that the chromosomes will be able to move to the metaphase plate (Gilbert, 2003).

Potential oocytes will go through meiosis up to the diplotene stage, but will remain frozen here until sexual maturation. These growing oocytes are still active

although they are not currently progressing through the cell cycle. Young oocytes in the diplotene stage have been known to transcribe genes for zona pellucida proteins (Gilbert, 2003).

Meiosis has the same basis stages that are present in mitosis, including prophase, metaphase, anaphase, and telophase. Just as in mitosis, during metaphase spindle fibers will form. These spindle fibers will pull the chromosomes apart during the next phase, anaphase. Then, during telophase, the cell membrane pinches off to form two distinct cells, completing cytokinesis (Figure 1) (Gilbert, 2003).

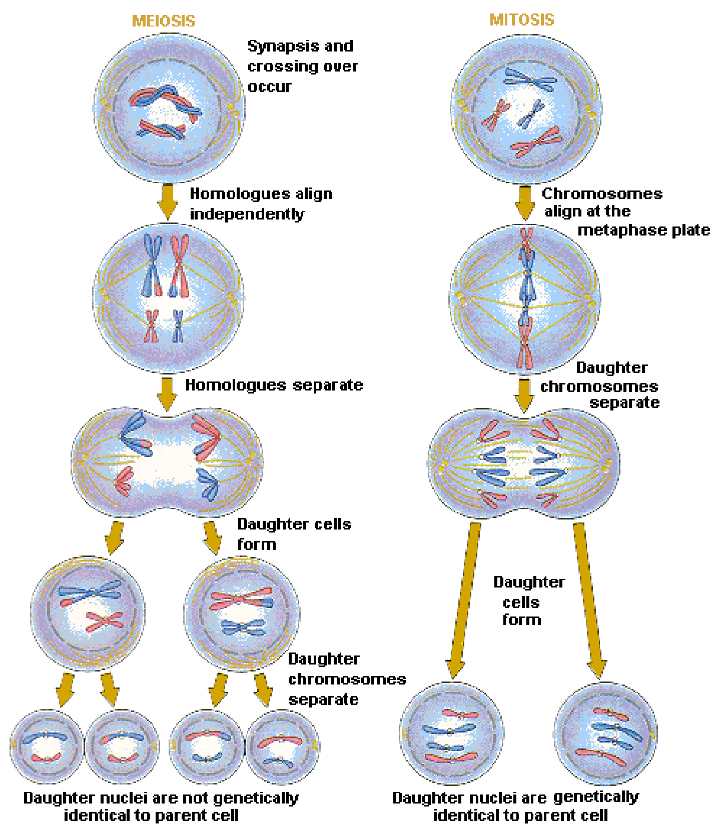


Figure 1. Meiosis and Mitosis Comparison (http://image.tutorvista.com)

To increase genetic diversity, homologous recombination, or the linking of homologs, will take place during prophase. Homologous recombination may lead to crossing over. Crossing over is strictly regulated in meiosis (Morgan, 2007). The determination of whether a chromosome will experience crossover or noncrossover recombination is controlled through independent pathways (Allers, 2001).

The synaptonemal complex is a ladder-like structure that facilitates synapsis. This synaptonemal complex contains two basic components: two lateral elements that correspond to the rails of a ladder and central elements that correspond to the rungs of a ladder. The lateral elements of the synaptonemal complex will bind to the chromatin to assist in pairing the homologous chromosomes (Schmekel and Daneholt, 1995). The synaptonemal complex may assist with crossovers (Morgan, 2007).

After meiosis I there is a brief interkinesis before meiosis II takes place. During metaphase II, spindle fibers will attach and chromosomes align in the center of the cell. In meiosis II anaphase the kinetochore splits, giving each cell two chromatids. Then, in telophase II the cell membrane pinches off and now there are four new haploid cells (Gilbert, 2003). According to Peping and Spradling (1998), there are so called "ring canals" that connect the daughter cells of meiosis I together through meiosis II. Vesicles and mRNA have been observed passing from one cell to the next.

During oogenesis, the first meiotic division will produce one cell, the secondary oocyte, that retains the cytoplasm, and the other cell gets little cytoplasm and is discarded as a polar body. The second meiotic division results in a second polar body, which

contains little more that genetic material, and the ovum, complete with ample cytoplasm (Gilbert, 2003).

Control of Meiosis

Meiosis regulation is very similar to the regulation of mitosis. Like mitosis, meiosis contains various checkpoints to control the progression of the cell through the cycle. These checkpoints are strictly regulated by the anaphase promoting complex (APC), Cdk-cyclin complex, and gene regulatory factors similar to those in mitosis. Unlike in mitosis, in meiosis, G1/S cyclins are inhibitors of meiosis. Also unlike mitosis, in meiosis, the reformation of the nucleus and the decondensation of the chromosomes at the end of meiosis do not have to occur to the level of completion that it does in mitosis. There is no checkpoint between meiosis I and meiosis II. So, when the cell commits to meiosis, it is committing to complete both phases of meiosis (Morgan, 2007).

Ime1 is a gene regulatory factor that has a large influence over promoting DNA synthesis and homolog recombination. When this regulatory factor is present there is an increase in expression of genes that influence the cell to go into the early phases of meiosis. The Ime1 gene is essential for the G1 to S phase transition (Benjamin, 2003; Morgan, 2007). Once the Ime1 gene is activated, it will target Ime2 to signal for DNA replication (Benjamin, 2003).

Ime2 is a protein kinase that has been found to be required for DNA replication during the early G1 phase of meiosis. Ime2 will decrease the concentration of Sic1, a Cdc28 inhibitor. Cdc28 is the major factor required for chromosome segregation in the later stages of meiosis. It is believed that both Ime2 and Cdc28 are needed throughout

meiosis for the G1 to S phase transition, for the G2 to M phase transition, and also for the progression through the M phase (Benjamin, 2003).

Methods for Studying

Methods for studying meiosis are basically the same as the methods used to study mitosis. The cell cycle stages can generally be determined through light microscopy. To aid in microscopic analysis, fluorescent labeling of various components of the cell can be applied (Morgan, 2007).

ENDOCRINE CONTROL OF OOCYTE DEVELOPMENT

Hypothalamic Regulation of Oocyte Development

Oocyte maturation and ovulation requires a balance of hormones (Etches, 1995). Gonadotrophin releasing hormone (GnRH) from the hypothalamus helps regulate the release of gonadortrophins, luteinizing hormone and follicle stimulating hormone, from the anterior pituitary. In the chicken, GnRH secretion is influenced by photo receptivity. An increase in the length of time of light exposure (daylength) increases the secretion of GnRH, which, in turn, induces an increase in the secretion of gonadotrophins from the anterior pituitary. This increase in gonadotrophins stimulates growth in ovarian tissue and helps to maintain a hierarchy of ovarian follicles. The hypothalamus also plays an important role in LH secretion, which influences ovulation. The portal vascular system connects the hypothalamus to the anterior pituitary, and it is through this route that GnRH travels to reach the anterior pituitary gland with its messages. While there are two forms

of GnRH present in the chicken, GnRH-I and GnRH-II, only GnRH-I is active in the signaling pathway between the hypothalamus and the anterior pituitary gland (Etches, 1995).

Role of Anterior Pituitary Gland (Gonadotrophins)

Gonadotrophic cells within the anterior pituitary gland react to pulses of GnRH by increasing their release of the gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Both LH and FSH are glycoprotein hormones, which share a similar α subunit held to unique β subunits by non-covalent bonding. It has been shown that LH is involved in inducing ovulation and also in stimulating steroid production in follicles. These surges in LH are controlled by circadian rhythms (Etches, 1984). The purpose of FSH in regard to follicular maturation is to amplify the effects of LH on ovulation (Kamiyoshi and Tanaka, 1972). While follicle stimulating hormone is thought to have very minor effects on the ovulation of follicles compared to LH, it has been shown to increase the DNA content of granulose cells supporting hierarchical follicles, which indicates proliferation of these cells (McElroy et al., 2004). Calvo and Bahr (1983) found that luteinizing hormone stimulates the adenyl cyclase activity of the largest hierarchical follicles while follicle stimulating hormone targets the smaller hierarchical follicles. Adenyl cyclase activity corresponds to the production and secretion of progesterone (Calvo and Bahr, 1983).

All of the follicles in the ovary are exposed to surges in plasma concentrations of these gonadotrophins and surges in steroid hormones secreted by ovulating and

developing follicles. However, only the hierarchal follicle is influenced by the signals (Etches, 1995).

Steroidogenesis by Ovarian Follicles

In the immature bird, the ovarian follicles begin to produce and secrete both androgens and estrogens, which will stimulate the onset of secondary sexual characteristics. In the immature follicle, the theca tissue will produce and secrete androgens, progestins, and estrogens. As the developing oocyte matures, progesterone production and secretion decreases until it is nonexistent close to the time of ovulation. Androgen production and secretion in the follicle increases when yolk begins to be secreted onto the oocyte. Its production peaks at the F_3 stage. However, within sixteen hours of becoming the F_1 follicle, the production and secretion of androgens by the follicle are completely terminated. Similar to androgen production, estrogens are produced by the follicle when it is recruited into the hierarchy, but is soon suspended. So, it seems that the follicle switches from the production of androgens to the production of estrogens between the F_n and the F_2 stages. However, progesterone production increases and peaks at ovulation. This peak in progesterone induces the hypothalamus to secrete GnRH. This causes the secretion of LH from the anterior pituitary (Etches, 1995). The LH signals the hierarchal follicle in the ovary to continue to produce progesterone. The progesterone, in turn, participates in the positive feedback system by encouraging the release of LH (Wilson and Sharp, 1976). This preovulatory surge of LH causes the synthesis of estradiol and androstenedione in the theca and granulose tissues in the follicle to end (Etches and Duke, 1984). It has been established that the follicle's

ability to respond to the LH surge with the production of progesterone is the element that determines that the follicle is ready to ovulate, not the size of the follicle (Etches et al., 1983). So, this surge in progesterone and LH causes the follicle to rupture and the egg to ovulate (Etches, 1995).

It has been suggested that the adrenal gland could have an influence over steroid production in the ovary. However, no proof exists at this time to support this theory. It has been shown that corticosterone, in combination with ACTH can cause ovulation of the mature follicle (Etches et al., 1982; Etches and Croze, 1983).

Estrogen stimulates the secretion of yolk materials onto the developing egg cell. Most of the precursors for the yolk materials originate in the liver. The estrogens stimulate these precursors to be transported to the ovary, and for the precursors to be deposited onto the oocyte. Most of this yolk deposition process occurs during the final ten days before ovulation (Etches, 1995).

The Ovulatory Cycle

Ovulatory cycles in chickens last from 24 to 28 hours, meaning that the chicken ovulates every 24 to 28 hours. Thirty to forty-five minutes after lying of the egg, the next oocyte ovulates. Luteinizing hormone is secreted from the anterior pituitary for eight hours out of the day. This secretion is controlled by circadian rhythms (Etches, 1995).

Four to six hours before ovulation, there is a surge in luteinizing hormone (LH), and four hours before ovulation there is a surge in progesterone, both of which are thought to trigger the maturation of the oocyte and ovulation (Doi et al., 1980; Johnson and van Tienhoven, 1980). Six hours before ovulation, a space between the maturing

oocyte and the perivitelline layer of the follicle is produced. In association with the production of this gap, the connective structures between the oocyte and the follicle begin to disintegrate (Yoshimura, 1993). It is thought that the surge in progesterone produced enzymes that aid in the breakdown of the follicle membrane (Doi, 1980).

CELL CULTURE

Cell culture is the isolation of individual cells for study. Because tissue culture was predominantly used to study cells for such a long period of time, the term “tissue culture” is still used to describe both organ and cell culture. The purpose of cell culture is to study characteristics of a particular type of cell, such as its cell-cell interaction, intracellular activity, environmental interaction cell products and secretion, intracellular flux, and genetics. Not only can scientists study the normal behavior and physiology of cells, but also researchers are able to study the effects of particular conditions and reagents on individual cell types using cell culture.

Cell culture offers numerous advantages to in vivo study of cellular interactions. One of the most important advantages is that cell culture offers an alternative to *in vivo* study. By utilizing cell culture, scientists can also control environmental factors, such as pH and temperature. Cell culture offers the ability to ensure homogeneity of cell types. Researchers can test reagents in cell culture using lower concentrations since the reagent is not circulated to other cell types

While cell culture is a wonderful tool that has abundant advantages, it is not without shortcomings. Cell culture requires a considerable amount of expertise in the technique to ensure a desirable outcome. Viruses, bacteria, and fungi can easily infect a

culture of cells if careful aseptic techniques are not utilized. Cell culture requires a great deal of effort to produce a relatively small amount of cells. In addition to these short comings, cells maintained in such an unnatural environment will lose some natural cell interactions and components. Good media and frequent changes of the media can help encourage natural behavior, but some natural behavior will be lost (Freshney, 1994) Cells can easily become overcrowded, exposing the cells to a build up of waste products and a shortage of nutrients.

Cells were dispersed in the nineteenth century, but it was much later that cell culture techniques were perfected. Sydney Ringer could be called the first to culture tissue because he developed a chloride solution that was capable of keeping a heart beating outside of a body (Ringer, 1883). Ross Granville Harrison vastly improved tissue culture techniques between the years of 1907 and 1910. Some of his techniques are still in use today (Harrison, 1907, 1910). Alexis Carrel is credited with developing methods to produce cell lines and then subculture these cell lines. Carrel's cell line derived from a chicken embryo heart was maintained from 1912 to 1946 (Carrel, 1911). There was a large interest in virology in the 1940s and 1950s that caused the development of cell culture techniques. As a result of this, animal cell culture was developed as a routine laboratory technique (Kuchler and Merchant, 1958; Cherry and Hull, 1956).

In 1916, Peyton Rous published a paper describing cell disaggregation through enzymatic dispersal. Rous was the first to use an enzyme to disassociate cells for cell culture. In this experiment, he used trypsin to separate cells from rat and chicken tissues (Rous and Jones, 1916). Enzymes are proteins that act as biological catalyst, facilitating chemical reactions. Enzymes have at least one active site, which is where they will

attach to a substrate. Enzymes are sensitive to pH and should be used as close to their optimum pH as possible. Enzyme action is also dependent upon temperature. Each enzyme has an optimul temperature. A substrate and enzyme will join together at a protein or active center on the enzyme (DeRobertis, 1965). Enzymatic dispersal and cell culture were used in this experiment to obtain isolated culture of chicken oocytes.

III. STATEMENT OF RESEARCH OBJECTIVES

The objective of this experiment was to: 1) Determine the feasibility of enzymatic dispersal and purification of immature avian oocytes; and 2) Determine whether flow cytometry can be used to analyze DNA content of isolated immature avian oocytes, and determine the cell cycle stage of oocytes in the immature ovary.

IV. MANUSCRIPT I

ISOLATION AND PURIFICATION OF OOCYTES FROM THE IMMATURE AVIAN OVARY

Abstract

The optimum concentration of collagenase for harvesting immature oocytes from the ovaries of three-week-old chicks was determined by testing three different concentrations of Type II collagenase. Three methods of removing contaminating non-oocyte cells from the cell solution were evaluated. It was found that increasing collagenase concentration increased the harvest of oocytes and that a combination of hypotonic lysing of erythrocytes and anti-fibroblast antibody attached to magnetic beads produced the best yield of purified oocytes.

Introduction

Dispersed chicken oocytes are needed for the advancement of their study. This experiment was conceived and completed to determine a sensible method of isolating immature chicken oocytes. The DNA content of the oocytes was also analyzed to determine their current position in the cell cycle. The timing of mitotic and meiotic events and the mechanisms that control them are not completely understood in the

chicken oocyte. Understanding these events could reveal a means of manipulating mitosis and meiosis to improve reproductive performance, and maybe even eventually reveal a way to control the gender of the offspring. The ability to harvest individual living oocytes from immature ovaries allows for culture and analysis of cell cycle events. It should be possible to stain the oocytes with fluorescent DNA stains and then analyze their position in the cell cycle.

Materials and Methods

All chicks used in this experiment were three-week-old broiler breeders. All of the animals were housed under similar conditions and with the same diet. The Auburn University Institutional Animal Care and Use Committee approved this experiment.

Enzymatic Dispersal of Ovarian Tissue

Three-week-old chicks were humanely euthanized via CO_2 inhalation. The ovaries of the female chicks were removed as cleanly as possible. The tissue was placed into sterile 50 ml test tubes, containing calcium/magnesium free Hank's Balanced Salt Solution (HBSS). The tissue samples were kept on ice to await culture. In a cell culture hood, the ovaries were washed in HBSS containing antibiotics and antimycotics. The ovaries were then weighed individually. The samples were divided into three tests, containing nine ovaries each. These groups were then divided into three isolates, each containing three ovaries. Each group of three ovaries was placed in Petri dishes and the tissue was cut into 2 mm chunks.

One of three different enzyme solutions was added to each group. The first enzyme mixture contained 30 mg Type 2 Collagenase, 15 mg Hyaluronidase, and 15 mg Protease. The second enzyme solution contained 60 mg Type 2 Collagenase, 15 mg Hyaluronidase, and 15 mg Protease. Finally, the third enzyme mixture contained 120 mg Type 2 Collagenase, 15 mg Hyaluronidase, and 15 mg Protease. Collagenase Type 2 used in this study was obtained from Worthington Biochemical, Lakewood, NJ. The Hyaluronidase and Protease used in this study were obtained from Sigma-Aldrich Co., St. Louis, MO. All enzyme solutions had a pH of 7.4 and were filter sterilized. The tissue samples with the enzyme solutions were placed into sterile 50 ml culture tubes and incubated in a 37°C shaking water bath for 45 minutes. At the end of the incubation period, the samples were gently triturated at a rate of about 3 ml/ second. The cell suspensions were then filtered through a 100 μm cell strainer. Filtrates were centrifuged at 1000 rpm for four minutes. The supernatants (enzyme solutions) were placed back into the tubes with the remaining chunks of tissue. The cell pellets were gently resuspended in 5 ml of M199 containing antibiotics and antimycotics. Cell suspensions were stored in the refrigerator. The remaining chunks of tissue were placed back in their respective enzyme solutions in the shaking water bath to incubate for an additional 30 minutes at 37°C. The solutions were then filtered through 70 μm cell strainers and the filtered chunks were discarded. The filtrates were centrifuged at 1000 rpm for 4 minutes. The supernatant was discarded and the cell pellets were resuspended in 5 ml M199 containing antibiotics and antimycotics. These cell suspensions were combined with the previous ones, and the combined suspensions were centrifuged at 1000 rpm for 8 minutes. The supernatants were discarded and the cell pellets were each gently

resuspended in 3 ml of M199 containing antibiotics and antimycotics. The cells were counted using a hemocytometer under a light microscope.

Purifying Oocytes

Three-week-old chickens were humanely euthanized via CO_2 inhalation. The ovaries were removed from the female chicks. The ovaries were placed in sterile 50 ml tubes in ice-cold calcium/magnesium free HBSS. The tissue samples were transported to the laboratory and washed in a cell culture hood with HBSS containing antibiotics and antimycotics. The ovaries were then weighed individually. The ovaries were divided into three groups, containing three ovaries each. One of which was be tested using red blood cell (RBC) lysing and fibroblast attachment, the second was tested using Percoll, and the third was tested using a combination of depletion of fibroblasts by antibody attachment and depletion of RBCs by RBC lysing. Each group was placed in a Petri dish and cut into 2 mm chunks. Twenty-five ml of enzyme solution, containing Type 2 Collagenase, Hyaluronidase, and Protease in 30 ml of HBSS was added to the tissue. This solution was placed into sterile 50 ml test tubes. The samples were then placed in a shaking water bath at 37°C for 45 minutes. At the end of the incubation period, the tissue chunks were gently triturated with a 10 ml pipette. The samples were then filtered through a 100 μm cell strainer. The filtrates were centrifuged at 1000 rpm for four minutes. The supernatants were combined with the filtered chunks of tissue. The cell pellets were resuspended in 5 ml of M199 containing antibiotics and antimycotics. These suspensions were then placed in the refrigerator. The remaining tissue chunks and the enzyme solution were placed back into the shaking water bath at 37°C for 30 additional

minutes. After the incubation period, the tissue was again filtered through a 100 μm cell strainer. The remaining tissue was discarded and the filtrates were centrifuged at 1000 rpm for 4 minutes. The supernatants were discarded and the cell pellets were resuspended in 5 ml of M199 containing antibiotics and antimycotics. These solutions were combined with the pervious cell suspension and the mixtures were centrifuged for 8 minutes at 1000 rpm. The supernatant was discarded and the cell pellets were resuspended in 9 ml of M199 containing antibiotics and antimycotics.   The cell suspensions were divided into 3 test tubes, each containing 3 ml of cell suspension. Each sample was counted using a hemocytometer.

Red Blood Cell Lysing with Attachment of Fibroblasts

A red blood cell lysing solution, containing 0.829 g Ammonium chloride (NH_4Cl), 0.1 g Potassium bicarbonate ($KGCO_3$), and 0.0037 g Ethylene diamine tetraacetic acid (EDTA) QS to 100 ml with DDH_2O was used in this experiment. The cell suspensions were centrifuged at 1000 rpm for five minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml of the RBC lysing solution described above. The cell suspensions were allowed to incubate for four minutes at room temperature, after which 6 ml of M199 containing antibiotics and antimycotics was added to the solution. The samples were centrifuged at 1000 rpm for five minutes to remove the lysing solution. The supernatant was discarded and the cell pellet was resuspended in 3 ml of M199 containing antibiotics and antimycotics. The oocytes were then counted in a hemocytometer. Seven ml of M199 containing antibiotics and antimycotics were added to the samples. The cells suspensions were placed in 25 cm^2 cell culture flasks and put

into a CO_2 incubator overnight. The cell suspensions were placed in 15 ml sterile test tubes and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 3 ml of M199 containing antibiotics and antimycotics. The oocytes were counted using a hemocytometer under a light microscope.

Percoll

A Percoll solution was made containing 37 ml Percoll, 3 ml 10x HBSS, diluted to 50% v/v with 1xHBSS. Three tubes containing 22 ml each of Percoll solution were centrifuged for 30 minutes at 37°C at 17,500 rpm. The cell suspension was concentrated by centrifuging the solution at 800 rpm for 8 minutes. The supernatant was discarded, and the cell pellet was resuspended in 2 ml 1x HBSS. The cell suspension was layered on top of the Percoll solution. The mixture was then centrifuged at 5000 rpm for 7 minutes at 37°C. The cell bands were observed. Five ml fractions were harvested and examined under a light microscope.

Red Blood Cell Lysing with Anti-Fibroblast Antibody Binding

A red blood cell lysing solution, containing 0.829 g Ammonium chloride (NH_4Cl), 0.1 g Potassium bicarbonate ($KGCO_3$), and 0.0037 g Ethylene diamine tetraacetic acid (EDTA) QS to 100 ml with DDH_2O was used in this experiment. The cell suspensions were centrifuged at 1000 rpm for five minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml of the RBC lysing solution described above. The cell suspensions were allowed to incubate for four minutes at room temperature, after which 6 ml of M199 containing antibiotics and antimycotics was added

to the solution. The samples were centrifuged at 1000 rpm for five minutes to remove the lysing solution. The supernatant was discarded and the cell pellets were resuspended in 1 ml of M199 containing antibiotics and antimycotics.

Dynabeads® Pan Mouse IgG were washed according to Invitrogen's instructions. The beads were pre-coated with an anti-fibroblast IgG antibody. The pre-coated beads were added to 1 ml of cell suspension and incubated for 30 minutes. The solution was exposed to a magnet for two minutes. The supernatant was poured off and examined in a hemocytometer.

Results and Discussion

Enzymatic Dispersal of Ovarian Tissue

As expected, the enzyme solution with the higher concentration of collagenase resulted in more oocytes in the cell suspension per gram of tissue. The enzyme solution with the lowest concentration of collagenase Type 2, which contained 30 mg Collagenase Type 2, 15 mg Hyaluronidase, and 15 mg Pronase, resulted in 3,472.22 cells/ml of oocytes per gram of ovarian tissue. The enzyme with the second lowest concentration of Collagenase Type 2, which contained 60 mg Type 2 collagenase, 15 mg Hyaluronidase, and 15 mg Pronase, resulted in 4,416.96 cells/ml of oocytes per gram of ovarian tissue. The enzyme solution containing the highest concentration of Type 2 collagenase, which contained 120 mg Type 2 collagenase, 15 mg Hyaluronidase, and 15 mg Pronase, resulted in 5,186 cells/ml of oocytes per gram of ovarian tissue

Purifying Oocytes

The red blood cell lysing solution worked quite successfully for removing contaminating red blood cells. There were no red blood cells present in the cell suspension after the lysing of the red blood cells. After incubating the cell suspension overnight in a CO_2 incubator to allow for fibroblast attachment, there was a noticeable decrease in the number of fibroblasts contaminating the cell suspension, however, not as drastic a decrease as desired. While this method of isolation seems to work quite well for removing the contaminating cells from the oocytes, there was a severe decrease in the number of oocytes remaining in the solution after such rigorous treatment. Immediately after dispersal, there were 7,053.61 cells/ml of oocytes per gram of ovarian tissue. After using the red blood cell lysing solution, there were 6,045.95 cells/ml of oocytes per gram of tissue. However, after incubating the cells overnight to allow for fibroblast attachment, there were only 2,015.32 cells/ml of oocytes per gram of tissue remaining. While it appears that this method of isolation can be very harsh on the oocytes themselves, it would be an ideal method if the researcher needed only a few oocytes to work with.

Using the Percoll density centrifugation gradient technique to purify the oocytes resulted in a large reduction in red blood cell contamination. However, there were still fibroblasts present, though significantly less so than before the treatment. The Percoll density centrifugation gradient technique led to a large drop in the number of oocytes present in the suspension. Their numbers dropped from 4,771 cells/ml per gram of tissue to 1,060.22 cells/ml per gram of tissue. This method of purifying the oocytes is more involved than the previous method, but has similar results. Both methods result in

eliminating the red blood cells present, but leaving some fibroblasts in the cells suspension, and lower the number of oocytes present. The Percoll density centrifugation gradient method did result in fewer fibroblasts in the cell suspension compared to the red blood cell lysing and fibroblast attachment.

The final method tested for purifying oocytes was the most successful. Lysing the red blood cells resulted in a drastic reduction in the number of contaminating red blood cells in the suspension. The anti chicken fibroblast antibody depletion resulted in a remarkable reduction in the number of fibroblast left in the cell solution. Oocytes in this experiment appeared to be healthy and viable after the treatment. Oocyte numbers dropped 16.67% from 12,048.19 cells/ml per gram of tissue to 10,040.16 cells/ml per gram of tissue. This reduction was the smallest reduction in oocyte numbers seen in this series of tests.

TABLE 1. Enzyme Concentrations

Enzyme Concentration	Mean Cell Count (cells/ml/gram of tissue)
Low Concentration 30 mg Type 2 Collagenase	1157 +/- 113
Medium Concentration 60 mg Type 2 Collagenase	1472 +/- 295
High Concentration 120 mg Type 2 Collagenase	1730 +/-499

n=3

TABLE 2. Oocyte Isolation

Method of Treatment	Cell Count Before Treatment (cells/ml/g tissue)	Cell Count After Treatment (cells/ml/g tissue)	Percent Reduction (%)
RBC lysis and fibroblast attachment	7,053.61	2,015.35	71.43
Percoll density centrifugation gradient	4,771	1,060.22	77.78
Anti-fibroblast Antibody Attachment and RBC lysis	12,048.19	10.040.16	16.67

FIGURE 2. Brightfield microscopy of isolated immature chicken oocytes

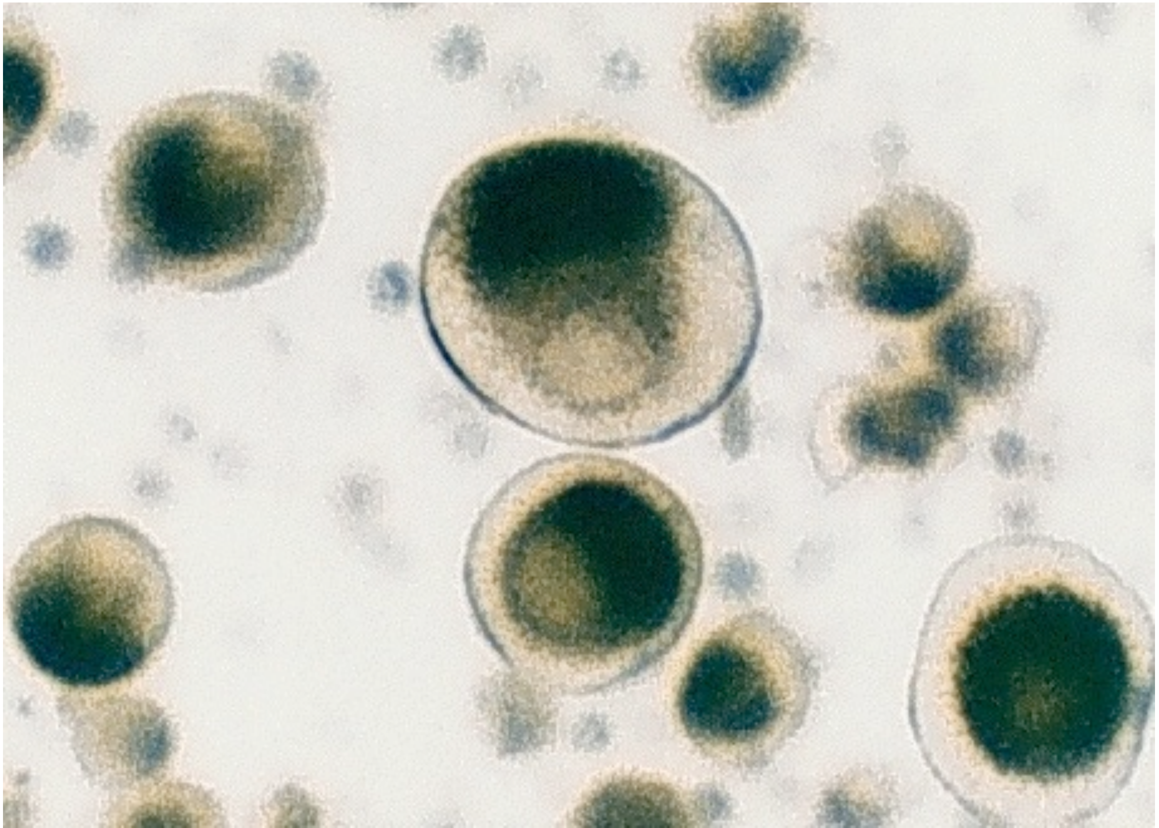


FIGURE 3. Darkfield microscopy view of immature chicken oocytes

FIGURE 4. Two oocytes accompanied by fibroblasts

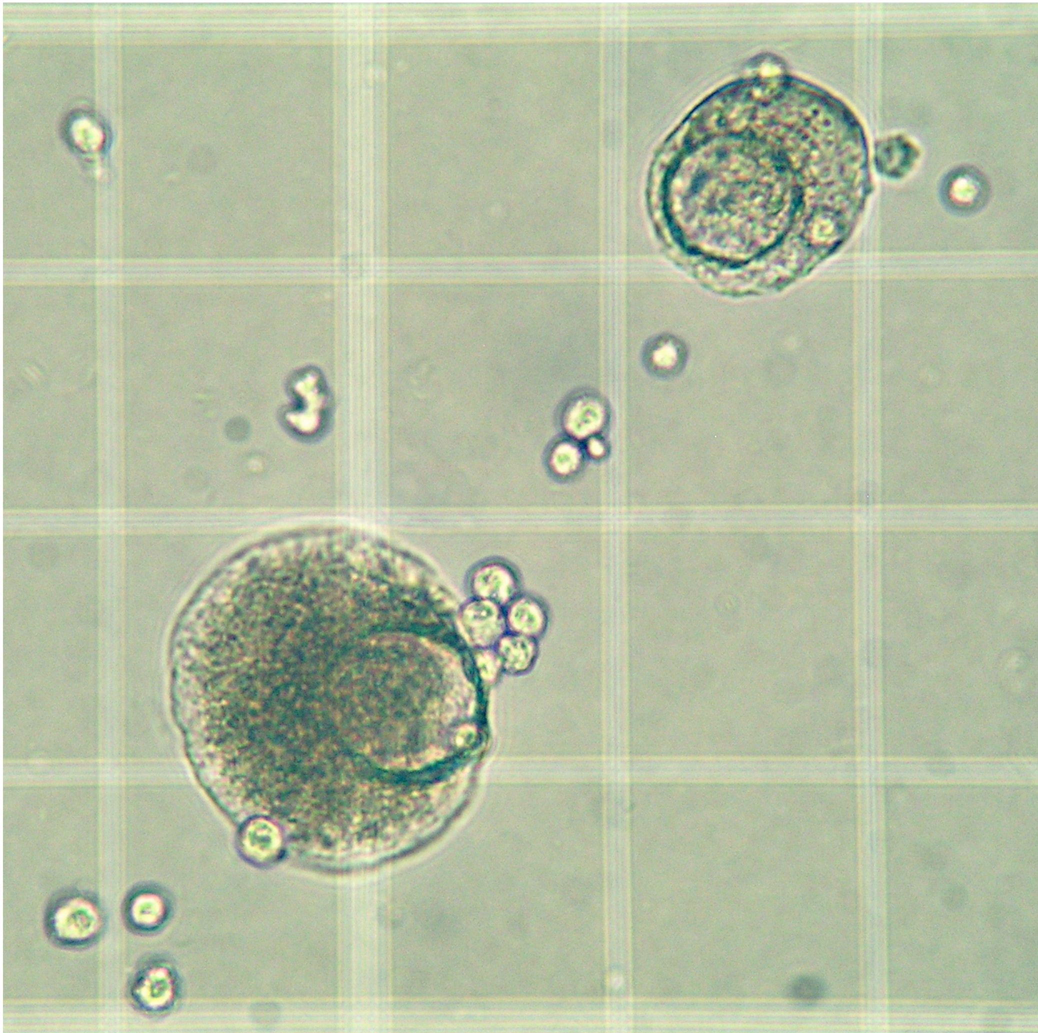
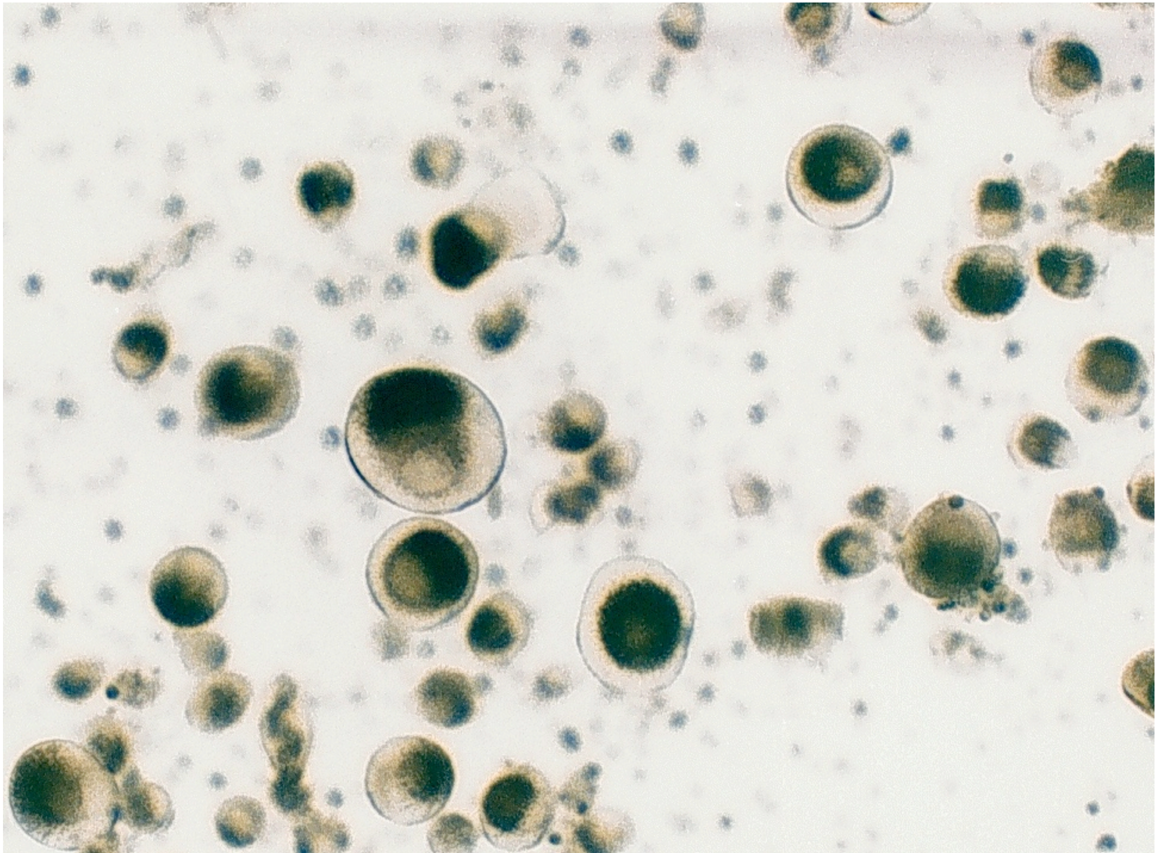


FIGURE 5. Cultured oocytes



V. MANUSCRIPT II

FLOW CYTOMETRIC ANALYSIS OF OOCYTE DNA CONTENT

Abstract

The cell cycle stage of three-week-old chicken oocytes was determined by the use of flow cytometry. It was found that flow cytometry can be used to analyze the cell cycle stage of oocytes. Typical flow cytometry equipment, techniques, and analysis was used to investigate the DNA content of immature chicken oocytes. This experiment suggests that the oocytes have not yet undergone meiosis and are arrested in a diploid state.

Introduction

In mammalian species, the male is heterogametic and thus determines the gender of offspring. The Y chromosome contains less DNA than the X chromosome, resulting in the potential "male" Y chromosome bearing sperm having less DNA than the potential "female" X chromosome bearing sperm. Using a flow cytometer equipped with a cell sorter, the amount of DNA in the sperm can be measured and the sperm separated into enriched X or Y bearing fractions (Gilbert, 2003; Johnson, et al 1993). These enriched sperm samples can then be used in artificial insemination programs to produce either female or male offspring as desired.

In avian species, it is the female that is heterogametic and determines the sex of the offspring. The female avian gametes contain both Z (equivalent to mammalian Y) and W (equivalent to mammalian X chromosome until they undergo meiosis II, just before ovulation (Etches, 1995; Pike and Petrie, 2003). The cumbersome yolk material secreted onto the egg cell in addition to the timing of the final, sex determining meiotic division make the avian oocytes more difficult to study. Currently there are no techniques to segregate the female avian gametes into "male" and "female" oocytes. Using isolated and purified avian oocytes, it should be possible to apply flow cytometric techniques to the oocytes to determine their DNA content, and effects of experimental manipulation on cell cycle events.

Materials and Methods

Harvested oocytes were centrifuged at 1200 rpm for five minutes. The supernant was then discarded and the cell pellet was resuspended in 1 ml of 70% ethanol to 1x106 cells while vortexing the cell pellet gently. The cell solution is fixed for thirty minutes at 4°C. The solution was then centrifuged again at 2000 rmp for five minutes. The supernant was discarded and the cell pellet was resuspended in 1 ml of phosphate buffered saline (PBS). The cell solution was centrifuged again at 2000 rpm for five minutes. This process of suspending the cell pellet in 1 ml of PBS and centrifuging was repeated two additional times. One hundred μl of ribonuclease (100 μg/ml DNase free, Sigma-Aldrich, Corp., St. Louis, MO) was added to the cell suspension. This mixture was incubated at room temperature for five minutes. At this time, 100 μl propidium iodide (50 μg/ml in PBS) was added to the solution.

Standard flow cytometry equipment was used in this experiment. The flow cytometer was a MoFlow (Dako, For Collins, CO). This flow cytometer was housed at the Auburn University College of Veterinary Medicine in Auburn, AL. The cytometric analysis was carried out be the resident flow cytometry technician. The flow cytometric data was analyzed using the FlowJo 8.8.6 software package (TreeStar, Inc., Ashland, OR)

It was determined that the three-week-old chick's oocytes have not undergone meiosis. This could allow for the exploration of factors that would influence the oocyte to enter into meiosis and possibly lead to advancements in reproductive efficiency and gender manipulation of the offspring.

Results and Discussion

All of the cells fall into one peak distribution (Figure 5), supporting the accepted theory that immature avian oocytes undergo meiosis at a later time. Figure 6 shows that all of the cells analyzed were the same type of cells. Since these oocytes have not undergone meisosis, it may be possible to explore the control of oocyte meiotic events in vitro. This could allow the identification of controlling factors, eventually leading to enhancement of reproduction and control of the gender of offspring.

FIGURE 6. DNA content vs. Number of Cells

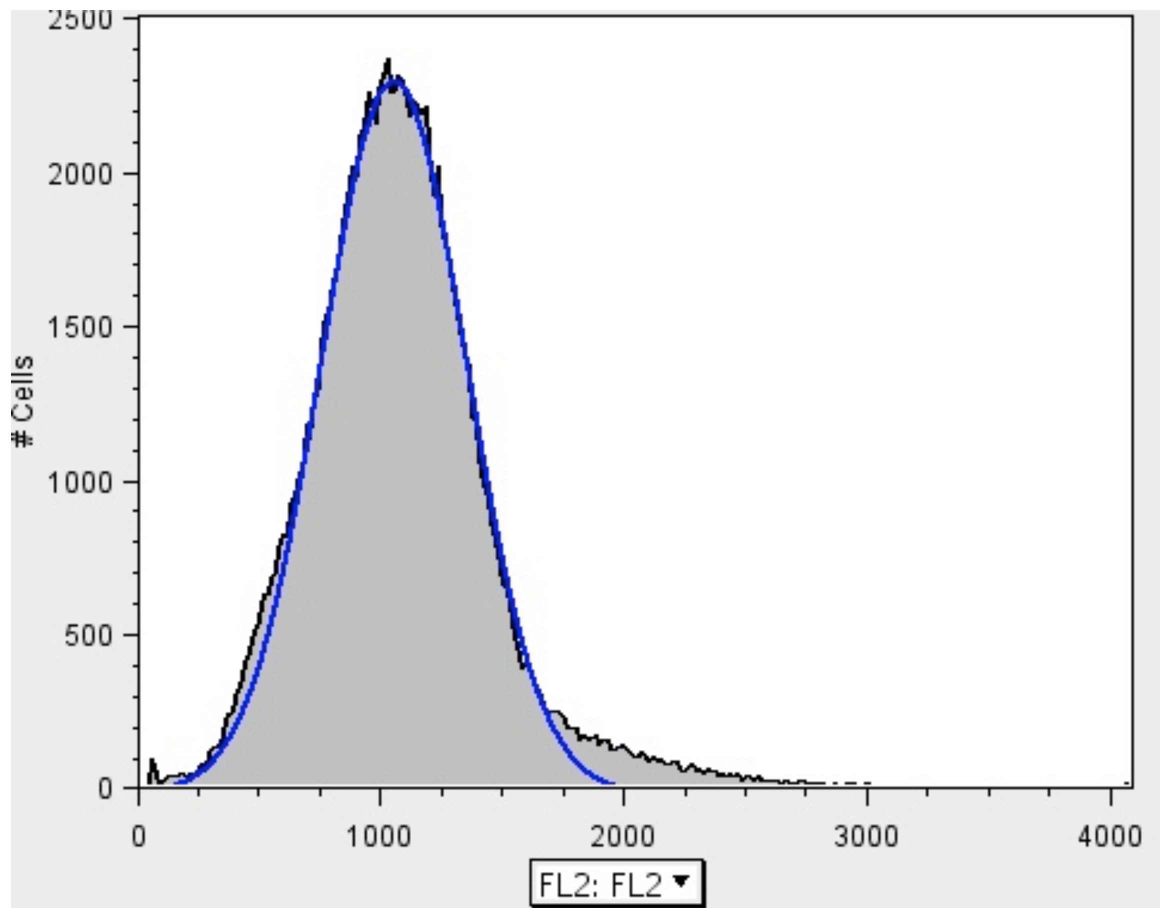
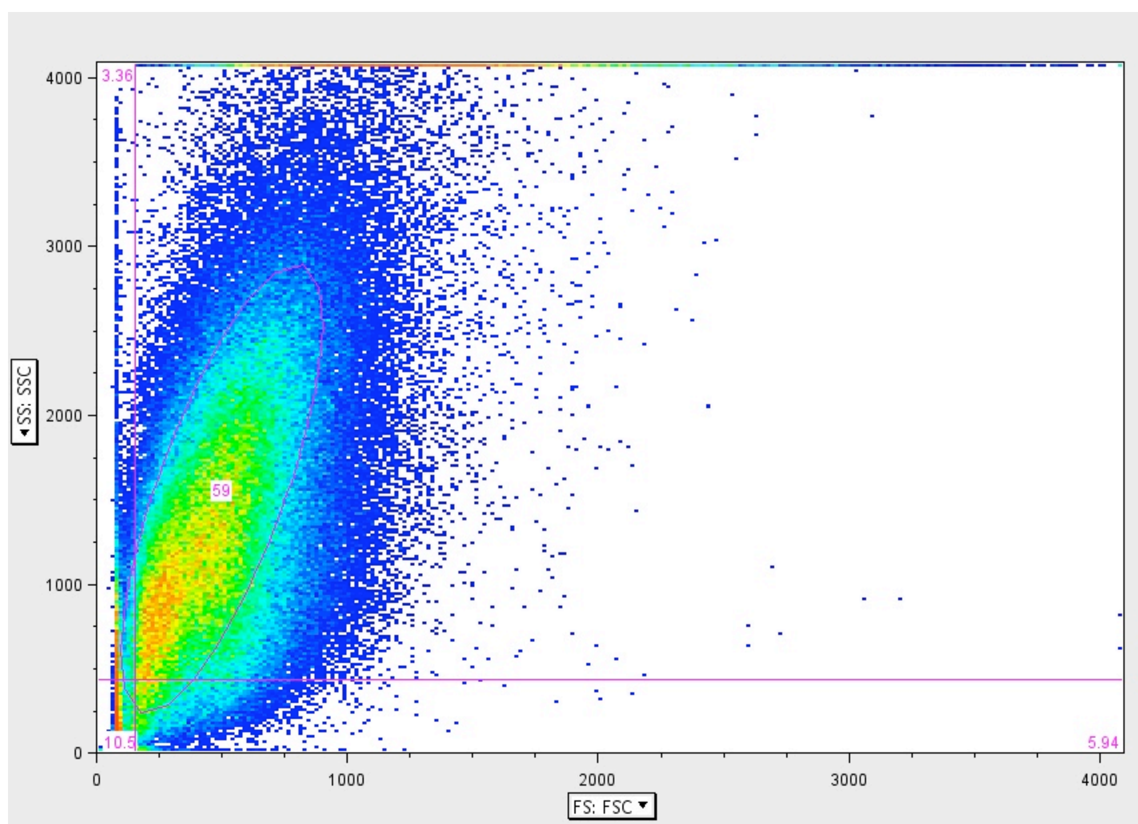


FIGURE 7. Forward Scatter vs. Side Scatter



VI. SUMMARY AND CONCLUSIONS

The objective of these experiments was to determine the feasibility of enzymatic dispersal and purification of immature avian oocytes, and also to determine the cell cycle stage of oocytes in the immature ovary. This experiment determined that it is feasible to disperse immature chicken oocytes using enzymes. It was also determined that purification of the oocytes could be achieved through lysing the contaminating red blood cells in an hypotonic solution, and then removing the contaminating fibroblasts with anti chicken fibroblast antibody depletion. Oocytes in chickens three weeks of age are diploid and have not yet undergone meiotic divisions.

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APPENDICES

Appendix 1

Red Blood Cell Lysis

There is an abundance of blood in the chicken ovary. Blood has to flow rapidly, and travel through capillaries that are smaller than the red cells themselves. Because of these demands, they posses a far more flexible membrane than other cells. Erythrocytes only have one cell membrane, unlike other cells. (Russel et al., 1982) The cytoskeleton of the red blood cell is also unique in that there are no filaments or tubules that span the length of the cell. The erythrocytes' cytoskeleton only contains proteins that run along the cell membrane surface. (Gunn, 1987) However, like other cells, erythrocytes' cell membrane is made up of proteins, lipids, and a small amount of carbohydrate.

This membrane is particularly sensitive to hypotonic solutions. (Russel et al., 1982) Solutions containing equal solute concentrations as the cell placed in them are said to be isotonic solutions. In an isotonic solution, the cell experiences a net movement of solution through the cell membrane. A hypotonic solution contains a lower concentration of solutes, and posses a lower osmotic pressure than the cell. When a cell is placed in a hypotonic solution, water passes through the cell membrane and causes the cell to swell. Cells can eventually swell so much that they burst in a hypotonic solution. A hypotonic solution can be made using inorganic salts, such as sodium or ammonium chloride. (Solomon, 2002) Because of the uniqueness of the red blood cell membrane and cytoskeleton structure, placing these cells in a hypotonic solution causes these particular cells to burst before more robust cells are affected. (Russell et al., 1982)

Procedure:

1.) Make the red blood cell lysing solution as follows:

Ammonium chloride (NH_4Cl) 0.15 M, 8.29 g

Potassium bicarbonate ($KHCO_3$) 10 mM, 1 g

EDTA 0.1 mM, 0.037 g

Distilled H_2O, 1 L

2.) Resuspend cell pellet in 1 ml red blood cell lysing solution
3.) Incubate at room temperature for four minutes
4.) Dilute solution in 6 ml M199
5.) Centrifuge at 1000 rpm for five minutes
6.) Discard supernatant
7.) Resuspend cell pellet in 3 ml M199 containing antibiotics and antimycotics

Appendix 2

Percoll Density Centrifugation Gradient

Procedure:

1.) Add 3 ml 10x Hank's Balanced Salt Solution (HBSS) to 37 ml Percoll and mix well

2.) Dilute the Percoll solution with 1x HBSS to 50% v/v

3.) Put 25 ml diluted Percoll in high speed centrifuge tube and centrifuge at 37°C for 30 minutes at 17,500 rpm.

4.) Concentrate the cell solution by centrifuging them at 800 rpm for eight minutes, remove the supernatant and resuspend the cell pellet in 2 ml HBSS.

5.) Carefully layer the cells on top of the Percoll by running them down the side of the tube very slowly.

6.) Centrifuge this mixture at 5,000 rpm for seven minutes at 37°C

7.) Remove the tube from the centrifuge carefully and examine the tube for cell bands.

8.) Harvest 5 ml fractions or individual cell bands, and examine each sample under a microscope for the presence of oocytes.

Appendix 3

Dynabeads® Pan Mouse IgG Separation Beads for Removing Fibroblast Protocol:

Procedure

1.) Washing the Dynabeads®:
   a. Resuspend the Dynabeads® in their vial by gently tilting the vial.
   b. Transfer 1ml of the Dynabeads® into a new tube
   c. Add 1 ml Buffer 1 to the tube. Put the tube on a magnet for 1 minute and discard the supernatant.
   d. Remove the tube from the magnet and resuspend the Dynabeads® in 1 ml Buffer 1

2.) Pre-Coating the Dynabeads:
   a. Transfer 20 µl Dynabeads® to a new tube
   b. Add 10 µl anti-chicken fibroblast antibody to the tube.
   c. Incubate on the spinner with gentle rotation for 30 minutes
   d. Place the tube on a magnet for 1 minute
   e. Discard the supernatant.
   f. Wash the beads again twice using 2 ml Buffer 1
   g. Resuspend beads in 0.1 μl Buffer 1

3.) Depletion of Target Cells:
   a. Place 1 ml cell solution in sterile tube
   b. Add 50 μl pre-coated Dynabeads® to the tube
   c. Incubate for 30 minutes on the spinner with gentle rotation at 2-8ºC for 30 minutes.
   d. Place the tube on a magnet for two minutes.
   e. Pull supernatant out of tube and into a new tube for additional observation or experimentation.
   f. Tube with beads can be discarded.

Solutions:

Buffer 1: PBS with 0.1% BSA, pH of 7.4

Appendix 4

Calculations:

Procedure:

Cell concentration per milliliter = Total cell count in 4 squares x 2500 x dilution factor
Cell concentration per gram of tissue = cell concentration/ weight of tissue

1.) Enzyme Concentrations:
  a. Low Concentration:

    Total weight of three ovaries = 4.92 g
    Average cell count = 17,083 cells/ml
    17,083 cells/4.92 g = 3,472.22 cells/ml/g tissue

  b. Medium Concentration:

    Total weight of three ovaries = 2.83 g
    Average cell count = 12,500 cells/ml
    12,500 cells/2.83 g = 4,416.96 cells/ml/g tissue

  c. High Concentration:

    Total weight of three ovaries = 2.89 g
    Average cell count = 15,000 cells/ml
    15,000 cells/2.89 g = 5,186 cells/ml/g tissue

2.) Red Blood Cell Lysing with Fibroblast Attachment:
  a. Before Treatment:

    Total weight of tissue = 0.827 g
    Average cell count = 5,833.33 cells/ml
    5,833.33 cells/0.827 g = 7,053.61 cells/ml/g tissue

  b. After RBC Lysing:

    Total weight of tissue = 0.827 g
    Average cell count = 5,000 cells/ml
    5,000 cells/0.827 g = 6,045.95 cells/ml/g tissue

  c. After Fibroblast Attachment:

    Total weight of tissue = 0.827 g

Average cell count = 1,666.67 cells/ml1, 666.67 cells/0.827 g = 2,015.32 cells/ml/g tissue

d. Percentage Lost:

7,053.61- 2,015.32 = 5,038.29
5,038.29/7,053.61 = 0.7143
71.43% of oocytes are lost during this procedure.

3.) Percoll Density Centrifugation Gradient:

a. Before Treatment:

Total weight of tissue = 0.786 g
Average cell count = 3,750 cells/ml
3,750 cells/0.786 g = 4,770.99 cells/ml/g tissue

b. After Percoll:

Total weight of tissue = 0.786 g
Average cell count = 833.33 cells/ml
833.33 cells/0.786 g = 1,060.22 cells/ml/g tissue

c. Percentage Lost:

4,770.99-1,060.22 = 3,710.77
3,710.77/4,770.99 = 0.7777
77.77% of oocytes are lost during this procedure

4.) Red Blood Cell Lysing with Anti-chicken Fibroblast Attachment:

a. Before Treatment:

Total weight of tissue = 0.415 g
Average cell count = 5,000 cells/ml
5,000 cells/0.415g = 12,048.19 cells/ml/g tissue

b. After Treatment:

Total weight of tissue = 0.415 g
Average cell count = 4,166.67 cells/ml
4,166.67 cells/0.415 g = 10,040.16 cells/ml/g tissue

c. Percentage Lost:

12,048.19 – 10,040.16 = 2,008.03
2,008.03/12,048.19 = 0.1666

16.66% of oocytes are lost during this procedure.