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STUDIES ON THE INTERACTIONS AMONG PORCINE OVARIAN FOLLICULAR CELLS

DISSEERTATION
Presented in Partial Fulfilment of The Requirements for The
Degree Doctor of Philosophy in The Graduate School
of The Ohio State University

By

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To my parents, wife, and children
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION ................................................................. ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS ..................................................... iii</td>
</tr>
<tr>
<td>VITA ................................................................................ iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS .......................................................... vi</td>
</tr>
<tr>
<td>LIST OF FIGURES .............................................................. viii</td>
</tr>
<tr>
<td>LIST OF PLATES ............................................................... x</td>
</tr>
</tbody>
</table>

| CHAPTER I. GENERAL INTRODUCTION ........................................ 1 |
| 1.1. Primordial Germ Cells ................................................... 1 |
| 1.2. Oogonia Period ............................................................ 2 |
| 1.3. Oocytenogenesis ........................................................... 3 |
| 1.4. Follicle Formation and Development ................................. 5 |
| 1.4.1 Primodial Follicle ........................................................ 8 |
| 1.4.2 Small Follicle .............................................................. 8 |
| 1.4.3 Medium Follicle ........................................................... 8 |
| 1.4.4 Preovulatory Follicle .................................................... 8 |
| 1.5. Kinetics of Follicular Development .................................. 9 |
| 1.6. Follicle Maturation ....................................................... 12 |
| 1.7. Oocyte Growth ............................................................. 16 |
| 1.8. Oocyte Maturation ....................................................... 18 |
| 1.8.1. Germinal Vesicle (GV) .................................................. 23 |
| 1.8.2. Germinal Vesicle Breakdown (GVBD) ............................. 23 |
| 1.8.3. Chromosome Condensation ........................................... 23 |
| 1.8.4. Metaphase II (Met II) .................................................. 24 |
| 1.8.5. Ovulation ................................................................. 25 |
| 1.8.6. Fertilization ............................................................... 27 |
| 1.9.7. Early Embryonic Development ..................................... 28 |

| CHAPTER II. EFFECTS OF PORCINE OOCYTE ON GRANULOSA CELL PROLIFERATION ................................................ 30 |
| 2.1. Abstract ................................................................. 30 |
| 2.2. Introduction ............................................................ 31 |
| 2.3. Materials and Methods ............................................... 32 |
| 2.4. Results ................................................................. 35 |
| 2.5. Discussion ............................................................. 36 |
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Effect of oocyte on mural granulosa cell proliferation in porcine</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of oocyte on cumulus cell proliferation in porcine</td>
</tr>
<tr>
<td>3.</td>
<td>Diagram shows the co-culture systems we used to study the interaction between porcine ovarian granulosa and theca cells</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of co-culture theca with granulosa cells on granulosa cell proliferation in porcine</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of co-culture granulosa with theca cells on theca cell proliferation in porcine</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of co-culture theca with granulosa cells on progesterone production by granulosa cells</td>
</tr>
<tr>
<td>7.</td>
<td>Effect of co-culture granulosa with theca cells on androstenedione production by theca cells</td>
</tr>
<tr>
<td>8.</td>
<td>Effect of interaction between granulosa and theca cells on the meiosis-arresting activity of granulosa cells</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of FSH on the meiosis-arresting activity of granulosa cells</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of FSH treatment of co-culture porcine granulosa and theca cells on the meiosis-arresting activity of granulosa cells</td>
</tr>
<tr>
<td>11.</td>
<td>Effect of progesterone (P₄) on porcine oocyte maturation</td>
</tr>
<tr>
<td>12.</td>
<td>Effect of dihydrotetosterone (DHT) on porcine oocyte maturation</td>
</tr>
<tr>
<td>13.</td>
<td>Effect of testosterone (T) on porcine oocyte maturation</td>
</tr>
</tbody>
</table>
14. Effect of steroids on porcine granulosa cell proliferation .............................................95
15. Effect of 0, 1, 5, 10 and 25 ng/mL human keratinocyte growth factor (hKGF) on porcine oocyte maturation .................................................................110
16. Effect of FSH on hKGF-induced oocyte maturation ...................................................112
17. Effect of 0, 1, 5 and 10 ng/mL hKGF on porcine granulosa cell proliferation .............114
18. Effect of FSH on hKGF-induced porcine granulosa cell proliferation .........................116
19. Effect of transforming growth factor-β1 (TGF-β1) on porcine granulosa cell proliferation .................................................................................................118
20. Effect of different hormonal combinations on spontaneous porcine oocyte maturation .....................................................................................................134
21. Effect of glycine on the cleavage rate of early embryonic development in porcine.............................................................................................................136
22. Effect of estradiol 17-β supplemented to in vitro maturation medium on the subsequent cleavage rate of the porcine oocyte matured and fertilized in vitro .............................................................................................................138
## LIST OF PLATES

<table>
<thead>
<tr>
<th>PLATE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Phase-contrast micrograph of porcine cumulus-oocyte complexes after collected from ovaries</td>
</tr>
<tr>
<td>II.</td>
<td>Phase-contrast micrograph of porcine oocyte at germinal vesicle stage</td>
</tr>
<tr>
<td>III.</td>
<td>Phase-contrast micrograph of porcine oocyte at metaphase I stage</td>
</tr>
<tr>
<td>IV.</td>
<td>Phase-contrast micrograph of porcine oocyte at metaphase II stage</td>
</tr>
<tr>
<td>V.</td>
<td>Phase-contrast micrograph of porcine early embryonic development stages</td>
</tr>
<tr>
<td>VI.</td>
<td>Phase-contrast micrograph of porcine early embryonic development stages</td>
</tr>
</tbody>
</table>
CHAPTER I

GENERAL INTRODUCTION

Life begins with fusion of male and female gametes. As a new life progresses the components of the next generation start to emerge. In females, the cycle of oogenesis is continuous beginning with formation of primordial germ cells (PGC), appearance of oogonia and oocytes, growth and maturation of follicles and ovulation of a small proportion of oocytes. Successful combination of male and female gametes and reappearance of the new PGCs begins the next generation of oogenesis. In this introduction, it is my intention to cover some aspects of female gamete formation and development, as well as, fertilization and early embryonic development. Histologically, germ cells which exit extragonadally are termed PGC. After germ cells are located within the gonads, they are referred to as oogonia in females. As an oogonium enters meiotic prophase, it is called an oocyte. The entire process from PGCs to oocytes can be divided into three major periods: PGC, oogonium and oocyte development (Siracusa et al., 1985).

Primordial Germ Cells

Primordial germ cells originate extragonadally from the dorsal endoderm of yolk sac and can be distinguished from surrounding somatic cells by special cellular granulosa and
chromosomes (Hilscher, 1983). These primordial germ cells eventually migrate from the dorsal endoderm of yolk sac to the genital ridges (Witschi, 1948). During PGCs migration, the population of these cells progressively increases (Tan and Snow, 1981). During porcine embryo development, PGCs were first observed on day 18 post coitum, however the germinal ridge (border of mesonephros) forms around days 24 or 25 post coitum (Black and Ericson, 1968).

**Oogonia Period**

Once germ cells colonized in the gonadal ridges, they are named oogonia and structurally very similar to PGCs (Siracusa et al., 1985). After migrating to the gonadal ridge, germ cells continue to proliferate mitotically. The number of oogonia peaks by day 50 in the porcine fetus (Black and Erickson, 1968).

Oogonia proliferation occurs in a synchronized pattern. Germ cells develop in clusters (Peters, 1978) and connected to each other by cytoplasmic bridges (Gondos and Zamboni, 1969; Russes, 1983). The cytoplasmic connections between germ cells result from incomplete separation of daughter cells during mitotic division. This incomplete cytokinesis appears after the germ cells arrive in the gonadal ridge and disappears after oocytes are enclosed by follicular granulosa cells. The number of intercellular bridges increases during the course of development. Once the pattern of incomplete cytoplasmic division is initiated, all subsequent divisions result in similar cellular connection. Thus, a large syncytium is created and the resulting interconnected cluster of cells differentiate as a unit (Gondos, 1973, 1978). Once the follicle forms, the intercellular bridges begins to disappear. The primary function of these cellular bridges is probably the synchronization of germ cell differentiation.
Once the number of germ cells per ovary reaches a peak value, this number starts to diminish by atresia (Black and Erickson, 1968) which continues throughout the entire reproductive life of an individual. In pigs, the normal gestation period is 114 to 115 days and the oogonia in the fetus have a high mitotic activity between day 20 to 50 of pregnancy. On day 50 of fetal life, germ cells number about a million and subsequently decline by day 90 to 100 in swine. From this point an oogonium is called an oocyte.

**Oocytogenesis**

Oocytogenesis is a phase of germ cell development from transformation of the first oogonium into an oocyte to the last oogonium's transformation (Siracusa et al., 1985). After a number of mitotic divisions, an oogonium leaves the proliferative pool of germ cells, enters meiotic prophase and is called an oocyte. Since all oogonia do not undergo this transition synchronously, both oogonia and oocytes can be observed in the same ovary over a long period. In Porcine fetus, the transition begins around day 40 post coitum and ends around day 10 postpartum (Black and Erickson, 1968). Although progression of oocytogenesis continues for 85 days, groups of clustered oogonia, syncytially connected by intercellular bridges synchronously enter meiotic arrest (Gondos, 1973, Peters, 1978).

The time an oogonium enters prophase arrest is considerably different between mammalian species, and is apparently affected by adjacent somatic cells of the ovary as well the intrinsic properties of the germ cells. Byskov et al. (1983) proposed that there were two types of meiosis in germ cells, delayed versus immediate meiosis. In species with immediate meiosis, such as mouse and primates, the onset of meiosis occur at the same time or shortly
after the fetal sex can be distinguished. The ovary is without apparent compartmentalization and absent of germ cell cords. In contrast, the delayed meiosis species, in cows, sheep, and pigs, germ cell cords form in the ovary at the same time as in the testis of male fetuses. The germ cells are packed with somatic cells and oogonia. During this period, sex steroids are produced in the ovary, especially estradiol (Grinsted et al., 1982). As the ovary differentiates, the cords become disorganized and loose in structure, which results in the initiation of meiosis (Byskov, 1979). Similar to the delayed meiosis in female, in testis, male germ cells are also packed with somatic cells forming primary then secondary germ cell cords. The primary germ cell cords of the testis are considered prerequisites for the prevention of meiosis (Byskove et al., 1983).

Somatic cells modulate entrance of germ cells into meiosis by specific secretions. Ovarian tissues, originated from mesonephros, produce meiotic-inducing substance (MIS) which induces meiosis whereas somatic cells in the fetal testis cords synthesize and release a meiosis-preventing substance (MPS) (Byskov, 1978; Grinsted et al., 1982). If the female germ cells are cultured with fetal mouse testis, meiosis is blocked. Conversely, meiosis can be induced in male germ cells of fetal mouse by ovarian tissue (Byskov, 1979).

The response of germ cells to MIS appears to be influenced by the sex chromosome constitution of the germ cells (McLaren, 1983). Male and female germ cells behave alike before they reach the genital ridge. However, once they colonize the gonadal ridge and undergo a few mitotic divisions, their developmental paths diverge. Following the last round of DNA replication, female germ cells enter meiotic prophase while the male germ cells become arrested in the G1 stage of the cell cycle.
After entry into the meiotic prophase, female germ cells undergo a second wave of degeneration, which is not only affected by intrinsic properties of oocytes, but also by the ovarian environment. Somatic cells appear to protect germ cells from degeneration as oocytes in the diplotene stage degenerate if they remain outside follicles (Ohno and Smith, 1964). Under culture conditions, survival rates of mouse oocytes are significantly improved if they are incubated on monolayer of follicle cells (Siracura et al., 1985).

Oocytes escaping from the second wave of degeneration will remain in the diplotene stage of meiosis. In most mammalian species, including pigs, the chromosomes retain a diplotene configuration (Baker, 1972). In this resting phase, oocytes persist until they either degenerate or resume meiosis before ovulation.

**Follicle Formation and Development**

As the ovary differentiates, somatic cell components of the ovary migrate around oocytes. As a result of this invasion, oocytes are surrounded by somatic cells. These non-germ cell components are derived from mesonephros. The mesonephros is the second of the three consecutive nephric structures (pre-, meso-, and metanephros), which develop consecutively during fetal life of all mammals. All three kidneys arise in the nephrogenic cord, which forms from the segmented intermediate mesoderm early in embryonic life (Peters, 1978). An oocyte surrounded by one layer of squamous cells is referred to as a primordial follicle. Ovarian stroma cells outside this basement membrane constitute the primary interstitium.
The squamous cells, or the pre-granulosa cells are derived from the mesonephric origin (Byskov, 1978), although cells of coelomic epithelium may also be involved (Merchant, 1975; Peters, 1978). Primary interstitial cells similarly originate from mesonephros tissue (Byskov et al., 1983). After the primordial follicles form, the follicle remains in a quiescent state for various period of time in G1 arrest.

In swine, the transitional phase from oocyte clusters to primordial follicles begins on day 60 post coitum and completed by 20 days postpartum (Oxender et al., 1979). These primordial follicles form a pool of small non-growing follicles.

Initiation of follicular growth occurs during fetal life in the farm animals and primates. Factors activating primordial follicles remain unknown. It has been postulated that the signal for initiation of follicular growth originates from the oocyte (Chang et al., 1976). Several lines of evidence indicate that the somatic cells (pre-granulosa cells) are unable to form a follicle in the absence of the oocyte (Merchant, 1975). Therefore, the arrangement of pre-granulosa cells within the primordial follicles may be under the control of the oocyte.

Interestingly, germ cells transplanted into the adrenal gland differentiate into oocytes and induce the surrounding cells to assume an orientational characteristic of pre-granulosa cells (Upadhya and Zamboni, 1982). Oocytes not only activate the primordial follicles, but also govern follicular maturation. Nekola and Nalbandov (1971) observed that the luteinization of granulosa cells can occur spontaneously in vitro but could be prevented by co-culture with oocytes. In vivo, follicles without an oocyte undergo luteinization in the absence of luteinizing hormone (LH) (Hubbard and Erickson, 1988). Coskun et al. (1995) reported that
Porcine oocytes secrete a factor(s) that inhibits cumulus and granulosa cell steroidogenesis in vitro. These data suggest that the oocyte may be an intra ovarian regulator of luteinization.

Information concerning the role of gonadotropin in initiation of primordial follicle growth is still controversial. Some studies suggest that gonadotropin may play a role in activation of the primordial follicles. For example, Mulheron et al., (1987) observed that before follicular cells begin morphological transformation, the cytoplasm and germinal vesicle of oocytes bind both follicle stimulating hormone (FSH) and LH. It is possible that gonadotropin (FSH, LH) activate oocytes first, and then trigger pre-granulosa cells to proliferate. Steroids may also be mediators of initiation of follicular growth. Lu et al., (1985) observed during pregnancy, or after prolonged high concentrations of progesterone, that activation of primordial follicles was retarded.

Following initiation of follicular growth, different sizes of follicles exist in the ovary. These follicles have been classified according to the number and layers of supporting cells around the oocyte, the presence of an antrum (Pedersen and Peters, 1968) or existence of granulosa cells (Hirshfield and Schmidt, 1987). In rats, a typical primordial follicle contains approximately 4 granulosa cells in the largest cross section (LCS). As all 4 cells undergo mitotic divisions, follicular cells will thicken to 8 cells and continue to proliferate. The follicle will double its cellular number in LCS with each advancing generation. A mature preovulatory follicle contains 2000 to 2500 granulosa cells in the LCS. At this time, the granulosa cells may be considered the 10th generation of descendants of the original 4 follicle cells that constitute the primordial cells (Hirshfield and Schmidt, 1987). In domestic animals, similar data are not available, therefore, follicles are typically divided into 4 classes according
to the descriptions of Pedersen (1970 a, b) in mice and Hirshfield and Schimdt (1987) in rats. The classifications are as following:

1. **Primordial Follicle.** The follicular cells are flattened in shape and arranged in a single layer around an oocyte. This is the first generation of follicle. The primordial follicles serve as the pool of oocytes available for recruitment throughout the reproductive life of the female. What triggers the recruitment of primordial follicle is unknown. It is believed that gonadotropin does not play any role in follicular growth until the antrum formation (Hafez, 1987)

2. **Small Follicle.** Following activation of the primordial follicles by unknown trigger, mammalian oocytes grow from 15-20 μm to 70-100 μm in diameter. This growth is first accompanied by a thickening of the squamous granulosa cells to cuboidal or polyhedral cells and proliferation of the granulosa cells. However, the oocytes still remain arrested in prophase of meiosis throughout their growth (Eppig, 1991). In this stage, follicular cells undergo 5 to 6 generations of proliferation, but the antrum has not formed yet. Outside granulosa cells, a theca layer appears in this stage. By the end of this stage, oocytes reach their full size.

3. **Medium Follicle.** An antrum is present in the follicle. This includes the seventh and eights generation of follicle. At this stage, a follicle is also called Graafian follicle.

4. **Preovulatory Follicle.** The antrum expands as follicular fluid accumulates. The oocyte is connected to the mural granulosa cells by cumulus oophorus. Follicular cells at this stage are highly differentiated and subdivided into regions of specialization (Zoller and Weisz, 1979). Theca cells are delineated into two layers.
During the course of follicular development, duration of cell cycles is affected by extra and intra ovarian factors. As a primordial follicle enters the growing pool, the granulosa cells have a long cell cycle for approximately two weeks. Subsequently, the generation time is shortened as the granulosa cells mature. The most rapid period of follicle growth occurs during the eighth generation with cellular number doubling in 24 hours. Afterwards, the proliferative potential of granulosa cells apparently becomes exhausted and the rate of mitotic divisions slows down dramatically (Hirshfield and Schmidt, 1987).

**Kinetics of Follicular Development**

Three physiological events have been involved in folliculogenesis; follicle recruitment, selection and dominance. The follicle recruitment has been applied to two situations; activation of primordial follicles (Goodman and Hodgen, 1983) or entry of small follicles into growth of medium follicles (Hirshfield, 1981). The term selection is used to indicate the final reduction of the group of medium follicles down to the size equal to the number of preovulatory follicles (Goodman and Hodgen, 1983). Selected follicles destined to ovulate are functionally dominant and inhibit unselected follicles from further development. These three events have been studied in monotocous species (cow, human), but, are not well described in polytocous species (rodents, sow).

After leaving the primordial follicle pool, developing follicles continue to grow until they reach their destination, either ovulation or atresia. Even though the rate of follicle development can be modulated by intra- and extra-ovarian factors, once follicles begin to proliferate, they will never return to resting phase again.
The number of follicles at any stages depends on three factors; the rate of entry of primordial follicles into proliferation, the rate of follicle growth and the rate of follicle reaching the final development.

In polytocous species, such as mice, rats, and pigs, only a single transition from small to medium to large follicles takes place during an estrous cycle. The factors that control differentiation of small follicles to medium follicles are also probably responsible for regulating the initiation of primordial follicle growth, as small follicles decrease coincident with medium follicle disappearance.

Contrary to polytocous species, monotocous species have a different form of folliculogenesis. Folliculogenesis appears to occur in a wave pattern. It was observed that two waves of follicular growth occur during an estrous cycle in cattle, one between day 3 and 12 and the other between day 12 and the subsequent estrus (Rajakoski, 1960). Matton et al. (1981) reported that growth and replacement of large follicles was more rapid at the end than at the beginning of the estrous cycle. They observed three waves of follicle growth in an estrous cycle, which took place on day 3 to 13, 13 to 18, 18 to estrus. This was further supported after ultrasonic examination of ovaries through the estrous cycle (Savio et al., 1988). The first dominant follicle was detected on day 4 of the estrous cycle, reached a maximum size on day 6 and disappeared by day 15. The second dominant follicle was noted by day 12, grew to full size on day 16 and became undetectable on day 19. The last one was identified on day 16, and reached the preovulatory size on day 21.

Apparently, follicle recruitment is different between the monotocous and polytocous species. In rodents, it starts after estrus and lasts until the middle of the estrous cycle. In
swine, this event begins right after ovulation and terminates on day 15 and 16 of the estrous cycle concomitantly with decline of serum progesterone and elevation of estradiol. However, follicle recruitment appears in waves in sheep and cow (Turnbull et al., 1977) throughout the estrous cycle.

It remains unclear what factors are involved in follicular recruitment. Possibly, gonadotropins play a role in recruitment. A sustained post ovulatory FSH surge occurs in hamster (Chappel and Selker, 1979), sheep (Goodman et al., 1981) and pigs (Van de Wiel et al., 1981; Kelly et al., 1988). This post-ovulatory FSH is associated with the rate of ovulation in the following estrous cycle (Chappel and Selker, 1979).

Following recruitment, follicle selection occurs. Follicle selection is defined as the period during which the fate of medium follicles is determined. After selection, medium follicles are destined to either continue maturation to ovulation or succumb to atresia. At the beginning of selection, granulosa cells in the medium follicles undergo their last two round of proliferation. Selection starts on day 16 in swine, at which time, the average number of granulosa cells is less than 30% of the number in preovulatory follicles on day 21 (Grant et al., 1989).

The factors segregating the follicles into one of the two destinations are still unknown. A cascade of endocrine events may associate hormonal changes with follicle selection. Follicle selection occurs on day 15 to 17 of estrus in gilts (Clark et al., 1982). During that period, progesterone drops precipitously, estradiol elevates rapidly and FSH decreases (Flowers et al., 1989). It has been proposed that gonadotropins are major components functioning in the follicle selection. By administration of exogenous FSH during the follicular
phase, superovulation occurs. Similarly, high concentrations of endogenous FSH also increase ovulation rates. Studies with pregnant mare serum gonadotropin (PMSG) indicates that FSH is involved in initiating growth of the primordial follicles, stimulating granulosa proliferation (sheep; Turnbull et al., 1977), reducing atresia of follicles (pigs; Guthrie and Bolt, 1989; rats; Hirshfield, 1989), inducing formation of antral cavity (Gore-Langton and Daniel, 1989) and promoting transformation of medium follicles to large follicles (mice; Halpin and Charlton, 1988).

Luteinizing hormone may be another component functioning in follicular selection and maturation. Taya and Sasamota (1989) reported that administration of LH induced ovulation during midpregnancy in rats, as happens in domestic species, and initiated the endogenous production of estradiol and inhibin in the medium follicle and eventually brings about ovulation.

Follicle atresia is the common fate of developing follicles. In rats, the 8th and 9th generations of follicles represent a crucial turning point in follicular development. Although follicles of all sizes can become atretic, most atresia occurs during the 8th and 9th generations of follicles (Hirshfield and Schmidt, 1987). Similarly, atresia frequently happens in relatively advanced stages in swine (Dufour et al., 1985).

Follicle Maturation

As follicles approach ovulation, the follicular cells are specialized structurally and functionally. Granulosa cells are differentiated into two major populations: mural granulosa cells and cumulus or corona radiata cells. Corona radiata are the granulosa cells immediately
adjacent to the oocyte and establish intimate contact through cellular processes transversing the zona pellucida or junctional gaps with the oolemma. These cells serve as nurse cells, providing nutrients and regulatory factors to the oocyte for its growth and maturation.

Cells lining the antral cavity are the majority of follicular cells and are called mural granulosa or parietal cells. Mural cells are primarily steroidogenic cells (Veldhuis et al., 1982). These granulosa cells of preovulatory follicles have differential ability in binding FSH (May et al., 1980; Fletcher and Greenan, 1985).

Like the gap junctions between corona radiata and oocyte, granulosa cells also form intercellular connection among themselves. These latter gap junctions were previously established in the primordial and small follicles (Funkenstein et al., 1980). The intercellular connections appear to act to coordinate follicular cell proliferation and differentiation (Fletcher et al., 1987). As granulosa cells differentiate, theca cells also organize into two layers (theca externa and interna) and acquire LH receptors and steroidogenic activity.

Biochemically, mature follicles are active steroidogenically, synthesizing large amounts of estradiol. Three major enzymes are required to synthesize estradiol in follicles, including cholesterol side chain cleavage cytochrome P-450 (P-450scc), 17-α-hydroxylase cytochrome P450 (P450-17), and aromatase cytochrome P450 (P450 arom).

Cytochrome P450scc is an enzyme converting cholesterol to pregnenolone, a rate-limiting step in progesterone synthesis. This enzyme is found in the mitochondria of steroidogenic cells as a part of a three enzyme system, including inducing ferredoxin (an iron sulphur protein) and ferredoxin reductase (a flavoprotein). Experiments with western blot techniques demonstrated that the amount of cytochrome P450scc present in preovulatory
folicules is greater than that in small antral follicles, but less than that in corpora lutea (Zlotkin et al., 1986). Cytochrome P450scc is only detected in the theca cells in well developed antral follicles, but not in the granulosa cells in bovine (Rodgers et al., 1986a) and human (Sasano et al., 1989).

The content of cytochrome P450scc mRNA is low in small follicles and high in preovulatory follicles, reaches a peak 7 h after LH surge and maintains this elevated level in mature corpora lutea in rats (Goldring et al., 1987). Cytochrome P450scc activity is affected by gonadotropin and steroids. Estradiol or FSH, administrated alone to rat granulosa cells in vitro and to hypophysectomized immature rats in vivo, does not change the quantity of mRNA for cytochrome P450scc. However, when FSH is given to estradiol treated granulosa cells or hypophysectomized rats, the amount of P450scc mRNA significantly increases.

The enzyme 17-α- hydroxylase cytochrome P450 (P450-17α) is a single peptide that contains two enzymatic functions, the 17-hydroxylase and C17-20 lyase activity, which converts progesterone to androstenedione. Like the distribution of cytochrome P450scc, P450-17 α is also only expressed in the theca cells. Its content is low in small follicles, high in preovulatory follicles and decreases in luteal tissue of rats (Hedin et al., 1987). These changes are associated with the alterations of mRNA content at different stages of follicular development (Hedin et al., 1987). These observations are similar to those reported by Rodgers et al. (1986 a, b), who demonstrated that cytochrome P450-17 was high in large bovine follicles but undetectable in luteal cells.

Theca cell differentiation is characterized by production of aromatizable androgens which are obligatory for follicular estradiol synthesis (Richards and Hedin, 1988). Based on
physiological studies in rats, it has been shown that slight increases in serum concentration of LH stimulate production of androgens, increasing in turn the available substrate to promote estradiol synthesis. This is consistent with the two cell theory of bovine steroidogenesis that theca cells convert progesterone to androgen and the latter diffuses to granulosa cells where the androgen is aromatized to estradiol (Fortune and Quirk, 1988).

Following the LH surge, estradiol production declines dramatically. This decrease has been shown to be related to the decline in cytochrome P450-17 content. Therefore, it appears that LH has biphasic effects on P450-17 activities. Low doses of LH increase cytochrome P450-17 content by stimulating its gene transcription. In contrast, high doses of LH inhibit the transcription and result in low enzymatic activities (Richards and Hedin, 1988).

Aromatize cytochrome P450 (P450arom) is exclusively localized in granulosa cells in rat ovarian follicles and is maintained by FSH in the presence of androgens (Richards and Hedin, 1988). In human, cytochrome P450arom, however, has been observed in the theca interna (Matsuda et al., 1984). By immunohistochemical staining, cytochrome P450arom is localized predominantly in the mural granulosa cells of well developed follicles during late follicular growth (Sasano et al., 1989). This observation is consistent with the findings that estradiol is synthesized in significant amounts only in preovulatory follicles. The cooperation of theca and granulosa cells is necessary because theca cells possess cytochrome P450scc and P450-17 to synthesize aromatizable androgens, and granulosa cells contain cytochrome P450arom to convert the androgens to estradiol (McNatty et al., 1979; Fortune and Quirk, 1988).
The endocrine events have extra-ovarian, intra follicular and oogenic significance.

LH appears to coordinate extra-ovarian events when released 2h after the onset of estrus in swine (Ziecik et al., 1987; Kelly et al., 1988).

Within the ovary, the hormonal changes might be important to augment oocyte maturation and follicular development.

**Oocyte Growth**

As a functional unit, the oocyte and its follicular cells develop coordinately (Pedersen, 1970 a, b). In sexually mature animals, oocytes complete growth before formation of a follicular antrum whereas follicular proliferation continues until right before ovulation or atresia.

Oocyte growth includes an increase in cellular volume nearly 300-fold (mouse: Wasserman, 1988), a change in appearance and a reorganization of some organelles (Zamboni, 1970, such as the nucleolus, mitochondria, Golgi complex, cortical granules, zona pellucida, cytoplasmic lattices and ribosomes). During oocyte growth, a large amount of RNA is synthesized and accumulated.

Oocytes grow concomitantly with their surrounding follicle cells. Ultrastructurally, follicular cells contact the oocyte by cytoplasmic processes with formation of heterologous gap junctions, provide channels for a direct passage of small molecules from follicles cells to the oocyte, such as energy substrates, nucleotidase, amino acids, (Heller and Schultz, 1980; Osborn and Moor, 1982), and mediate metabolic cooperativity (Moor et al., 1980).
Intracellular communication between the oocyte and follicle cells apparently is essential for mammalian oocyte growth. Studies in vitro demonstrate that denuded oocytes (Follicular cells-free oocytes) grow very little, if at all. Denuded oocytes become atretic after several days in culture. In contrast, mouse oocytes continue to grow in vitro when intracellular communication with cumulus cells is maintained (Eppig, 1977) or is re-established in vitro (Herland and Schultz, 1984). For example, cumulus-enclosed fully grown oocytes (Heller and Schultz, 1980) and follicle-enclosed growing oocytes (Heller and Schultz, 1980) take up significantly more uridine, guanosine and choline from culture medium than do denuded growing or grown oocytes. Since growing oocytes lack the energy-dependent A-transport system for uptake of certain amino acids, it is possible that follicle cells provide these amino acids to oocytes through gap junctions (Colonnanan and Mangia, 1983).

Besides supplying nutrients, gap junctions also regulate oocyte growth, even though it has been demonstrated that mouse oocytes can grow on monolayer of follicle cells (Bachvarova et al., 1980). Coskun and Lin (1993) reported that epidermal growth factor (EGF) enhanced the nuclear maturation of porcine oocytes enclosed in cumulus cells, but that did not happen in denuded oocytes. These observations suggest that besides supplying nutrients, follicle cells provide the oocyte with some specific factors required for oocyte growth and maturation via gap junctions. As oocytes enlarge in volume, the extent of metabolic cooperativity increases, and the rate of growth in vitro is dependent on the extent of intercellular communication (Brower and Schultz, 1982; Buccione et al., 1987).
Oocyte Maturation

Oocyte maturation is a process of the conversion of fully grown oocytes into fertilizable ova at ovulation. In particular, meiotic maturation refers to nuclear advancement from diplotene stage of prophase to metaphase II, including exclusion of the first polar body.

In mammals, the size of oocytes is correlated with the capacity to resume meiosis. Porcine oocytes in a follicle larger than 1 mm in diameter acquire the competence to resume meiotic division \textit{in vitro}, but only those of follicles about 2 mm in diameter complete the first meiotic division \textit{in vitro} (Motlik et al., 1984). These results suggest that acquisition of meiotic maturation occurs in two steps. For example, growing oocytes first attain the ability to undergo germinal vesicle breakdown, and continue to metaphase I, followed by acquisition of the capacity to progress from metaphase I to metaphase II. (Wassarman, 1988).

Oocytes of healthy mammalian follicles are arrested in meiotic prophase prior to the preovulatory surge of gonadotropin. However, when oocytes were liberated from antral follicles and cultured in vitro, they resumed meiotic divisions spontaneously Pincus and Enzmann, 1935). This observation provoked the hypothesis that antral follicles held oocytes in prophase arrest until after the preovulatory surge of gonadotropin.

Three types of molecules have been postulated to take a part in suppression of meiotic progression: cAMP (Eppig, 1985), follicle steroids (McGaughey, 1977) and oocyte maturation inhibitors (Tsafriri, 1985). Spontaneous maturation of mouse oocytes does not occur \textit{in vitro}, for example, under those conditions which culture oocytes in the presence of either membrane-permeable analogs of cAMP or phosphodiesterase inhibitors (Racowsky, 1985). In addition, agents that increase intracellular cAMP levels by activating adenylate
cyclase also suppress the spontaneous oocyte maturation in vitro (Sato and Koide, 1984). However, micro injection of phosphodiesterase into oocytes cultured in vitro in the presence of phosphodiesterase inhibitors overcomes the inhibitory effect of phosphodiesterase inhibitors on meiotic divisions (Bornslaeger et al., 1986a). These observations imply that cAMP may participate in maintaining oocytes at the diplotene stage.

Involvement of cAMP in meiotic arrest may act via cAMP dependent protein kinase which contains catalytic \( \alpha \) and regulatory (R) subunits. In the absence of cAMP, the enzyme is inactivate with the two subunits binding together. In the presence of cAMP, R subunit binds to cAMP. This binding subsequently results in dissociation of R from C subunit, and activates the cAMP dependent protein kinase subunit C which phosphorylates oocyte proteins. Continuous phosphorylation of oocyte proteins maintains oocytes in meiotic arrest. Intracellular cAMP can control meiotic maturation by regulating the amount of free C subunit. If the free C subunits are low, phosphoprotein will be dephosphorylated and meiotic maturation will be assumed. Consistent with this hypothesis, micro injection of free C subunits keeps oocytes in meiotic arrest (Bornslaeger et al., 1986a, b).

Cumulus-oocyte complexes are comprised of cumulus cells and oocytes that are morphologically and functionally associated with each other through heterologous gap junctions (Albertini and Anderson, 1974). It has been reported that communication via gap junctions between cumulus cells and oocytes provides avenues for the bidirectional transfer of information (Downs, 1993). Two theories concerning the relationship of gap junctions to meiotic maturation have been proposed. The first theory is that gap junctions may mediate the transfer of inhibitory substances from cumulus cells to the oocyte (Sherizly et al., 1988).
The second theory suggests that a positive stimulus is generated by cumulus cells in response to hormones, and this stimulus is then transferred into oocytes via gap junctions to stimulate maturation (Downs et al., 1988). Intercellular connections between the oocytes and cumulus cells is also involved in maintenance of the intracellular concentrations of cAMP in oocytes. Bornslaeger and Schultz (1985 a, b) reported that FSH, which activates adenylate cyclase by ribosylation of C subunits, elevated cAMP concentration in oocytes in cumulus-oocyte complexes, and apparently, the increased cAMP in oocytes was transferred from cumulus cells via gap junctions. Evidently, disappearance or functional interruption of gap junctions is temporally correlated with germinal vesicle break down (GVBD) and cumulus expansion (Dekel and Poitkewitz, 1989). Just before ovulation, the number of gap junctions drops significantly and the net area of cumulus cell gap junction membrane decreases about 15-fold (Larsen et al., 1986). The oocyte and the surrounding cumulus cells are no longer metabolically coupled as a result of mucification and cumulus expansion (Dekel et al., 1978). These observations may explain spontaneous maturation of oocytes in vitro after removal of the inhibitory influence of follicle cells from the oocyte.

Following the LH surge, steroidogenic activity also alters dramatically. Estradiol decreases rapidly while progesterone rises at this time. Changes are speculated to be associated with oocyte maturation. The effects of steroids on nonmammalian oocyte maturation is well recognized. Progesterone stimulates meiotic maturation in amphibian oocytes. It has been shown that gonadotropins induce oocyte maturation in the presence of, but not in the absence of, cumulus cells. Gonadotropin may stimulate the synthetic activity of the cumulus cells, and thus indirectly induce maturation of oocytes. Evidence indicated that
the stimulatory effect of gonadotropin on oocyte maturation can be abolished in the presence of protein synthesis inhibitors (Petrino and Schuetz, 1986 a, b). Apparently, newly synthesized proteins are associated with the mitochondria side chain cleavage systems. In addition, progesterone can mimic the effect of gonadotropin on the oocytes in vitro by promoting maturation. If the synthetic activity of the somatic cells is interrupted, induction of oocyte maturation by gonadotropin will be abolished, but by progesterone remain effective (Schuetz, 198). Therefore, regulation of oocyte maturation by gonadotropin can be viewed as a two-step event: gonadotropin turns on the genes required for progesterone synthesis, which in turn, promote the newly synthesized progesterone to induce oocyte maturation.

In contrast to nonmammalian species, progesterone has been considered inhibitory on, or lacks involvement with, oocyte maturation (Kaji et al., 1987; Wassarman, 1988). This consideration is derived from experiments in which selected steroids, at various doses, were examined regarding their ability to overcome suppressive effects of inhibitors on spontaneous oocyte maturation in vitro. The most extensively used inhibitors are cAMP derivatives.

Some experiments more strongly suggest that steroids are major components involved in regulation of oocyte maturation. It was observed that ovine embryos would not develop normally after transfer to recipients if the oocytes were removed from follicles matured in vitro and fertilized in vivo (Moor and Trounson, 1977). The follicle tissues influence oocyte maturation at different levels: the zona pellucida, the oolemma and the cytoplasm. They promote oocyte maturation on egg penetrability and pronuclear formation (Mattioli et al., 1988). The effect of somatic gonadal cells appears to be mediated by soluble molecules, including progesterone and estradiol. Progesterone increases the penetrability of the oocytes
while estradiol decreases polyspermy by inducing polyspermy-block mechanism (Karlach, 1987). Proper steroid hormone environments also keep oocytes from abnormal development.

Maturation of the oocyte cytoplasm is also dependent on follicular steroid environment. Exposure of follicle to aminogluthimide, an inhibitor of cholesterol conversion to pregnenolone, reduced steroid production in the follicles, and subsequently, the follicle-enclosed oocytes formed abnormal pronuclei after fertilization (Moor et al., 1980). Replacement of steroids in this model increased the number of oocyte with normal pronuclei. This observation indicates that steroids actively participate in preparation of the oocyte cytoplasm for zygotic development.

The mammalian oocytes and their surrounding cumulus cells are metabolically coupled through gap junctions which provide the unique means of entry into the ooplasm for several metabolites (Moor et al., 1980). This intercellular coupling gradually decreases during the maturation of the oocyte, probably due to the influence of gonadotropin. The traditional concept that the dissociation of gap junctions results in the resumption of meiosis during the preovulatory stage has been challenged. Downs et al. (1988) suggested that the effect of FSH on maturation of cumulus-enclosed mouse oocyte was inhibitory at first, and later, it became stimulatory. In contrast, epidermal growth factor stimulated germinal vesicle breakdown at any time. Apparently, the induction of meiotic maturation is not mediated solely by oocyte-cumulus cell uncoupling, nor termination of the transfer of suppressive molecules from the cumulus cells to oocytes, but also a positive signal may be generated by cumulus cells in response to FSH or epidermal growth factor treatment (Downs et al., 1988; Coskun and Lin, 1993).
The sequence of meiotic maturation starts with germinal vesicle breakdown (GVBD) and is followed by diakinesis, metaphase I (M I), anaphase I (Ana I), telophase I (Telo I), and metaphase II (Met II). After oocytes reach Met II, they remain arrested again until fertilization.

Morphologically, these meiotic stages in pigs can be observed microscopically (Motlik and Fulka, 1976) and classified into the following stages.

**Germinal Vesicle (GV):**

The intact germinal vesicle is observed in fully grown oocytes which still remain at the diplotene stage. The large nucleus is located peripherally and contains fine granular materials. The nuclear envelope is intact. Chromatin is stained only around the nucleus in the form of a ring or horseshoe. The nucleoplasm is finally granular.

**Germinal Vesicle Breakdown (GVBD):**

This stage of oocyte development includes resumption of meiosis and progression to diakinesis. The first sign of GVBD is appearance of orcein-positive zones and is followed by disappearance of nuclear membrane and nucleolus. The chromatin is seen either as an irregular network or the individual filamentous bivalent. Later, the chromatin undergoes condensation into a single lump or into small discrete fragments.

**Chromosome Condensation:**

During meiotic maturation, oocyte chromosomes (bivalent) pass through metaphase I, anaphase I, and telophase I, arresting at metaphase II without an intervening prophase II. Diffuse dictate-stage chromosomes (resembling “lampbrush” chromosomes) undergo significant condensation along the inner margin of the nuclear envelope, concomitant with the
envelop's undulatory behavior (Wassarman et al., 1976). At metaphase I (Met I), the bivalent of chromosomes lie in an orderly sequence on the equator of a bipolar spindle. At anaphase I (Ana I), of the two triangular shaped chromosome groups, one moves to the periphery to become the first polar body, the other remains in the oocyte proper. The stretched appearance of bivalent undergoing disjunction is characteristic of Ana I. While in the early telophase I (Telo I) two dense chromatin groups are noted. The chromosomes of the first polar body are similar to those of the oocyte. In late Telo I, the polar body is formed and its chromosomes have scattered. The chromosomes of the oocyte are shaped like a half-moon with flattened portion directed toward the polar body and the convex side toward the egg interior.

**Metaphase II (Met II):**

The chromosomes are distributed as in the Met I except that the chromosomes are thinner and less extended compared to the chromosomes in Met I.

Processes of meiotic maturation in swine oocytes have been studied both *in vitro* and *in vivo* (Hunter and Polge, 1966; Hunter, 1974; Ainsworth et al., 1980; and Motlik et al., 1984). The estimation of duration from one meiotic stage to next varies considerably between researchers. This variation may be attributed to different conditions under which the estimation is made. For example, animals of different breed, age and physiological states were utilized for those studies. The experimental conditions, moreover, might create greater variability in the estimation, such as natural versus induced maturation *in vivo*, maturation *in vivo* versus that *in vitro*. Even by extrapolating the data accumulated during the last three
decades, still the definite intervals from one stage to another in meiotic division are very difficult to measure,

Ovulation

Initiated by the LH surge, ovulation is the culmination of a series of events; resumption of meiosis, luteinization of follicular cells, rupture of follicles and release of the oocyte. The process is associated with follicle hormone changes and has been speculated as an inflammatory reaction (Espey, 1980)

The steroidogenesis of follicular cells changes dramatically as the LH surge stimulates cytochrome P450 scc gene transcription and inhibits the P450 17 gene expression (Goldring et al., 1987). As a result of enzymatic alterations, follicular concentrations of androgens and estrogens decrease rapidly. However, progesterone increases (Callesen et al., 1986). Progesterone is suggested to participate in the ovulation process by enhancing follicular activity of prostaglandin E2-9-Keto reductase which converts PGE2 to PGF2α (Murdoch et al., 1986). PGE2 appears to be involved in the dissociation of follicle cells during ovulation. Inhibition of PG synthetase prevents dissolution of cell-to-cell contacts, but this inhibitory action can be reversed by PGF2α (Murdoch, 1988).

Accompanying the alteration in steroidogenesis, prostaglandin biosynthesis is elevated in follicular cells of periovulatory follicles. Like plasminogen activators and collagenase, prostaglandins (PGs.) are obligatory for the ovulation process (Espey et al., 1980 and Murdoch et al., 1986). In the mature follicles, the LH surge activates adenylate cyclase and a resulting increase in cyclic adenosine monophosphate (cAMP), which stimulates biosynthesis of PGs (Murdoch, 1985). In swine, the site of biosynthesis of PGs changes as
follicles differentiate. Prior to the LH surge, theca cells are major sources of PGs. Following the LH surge, both theca and granulosa cells markedly increase biosynthetic activity. In particular, granulosa cells magnify their production of PGE$_2$ 10-fold and PGF$_2\alpha$ 30-fold (Tsang et al., 1989). The response of granulosa and theca cells to the LH surge is limited to the preovulatory follicles, but not to the small antral follicles (Rhodes and Inskeep, 1988).

Ovulation is the consequence of a cascade of hormonal events orchestrated by the LH surge. High doses of LH stimulate specific steroidogenic enzymes of preovulatory follicle to synthesize progesterone and enhance the PGs synthetase activity. As a result, the increased progesterone and PGs stimulate plasminogen activator and collagenase production. Together, these products cause a remarkable change in ovarian vasculature, referred to as hyperemia. The hyperemic response of the ovary is attributed to an increase in blood flow and accompanied by vasodilation, edema, increase in vascular permeability and extravasation of blood into the peripapillary stroma (Espey, 1980). Plasminogen activators then convert the plasminogen in follicular fluid and tissue to plasmin (beers et al., 1975). Plasmin acts on collagen attached to collagen fibers. Local increases in amount of collagenase and serine protease then hydrolyze the collagen. As a consequence, tensile strength of the follicle wall is reduced to such extent that existing intra follicular pressure can rupture the follicle (Lipner, 1988).

The LH surge is initiated by a rapid rise of estradiol in follicular phase in gilts. The preovulatory surge of LH initiates the ovulation process. Following peak concentration of estradiol in blood, gilts come to standing estrus about 22 h later (Ziecik et al., 1987). Ovulation occurs 34 h after the estrus (Pope et al., 1988).
Fertilization

Following ovulation, oocytes are recovered by fimbriae and transported to the ampullary-isthmus junction. At this location the oocytes interact with precapacitated sperm and fertilization takes place. Transportation of oocytes to the site of fertilization takes about 30 to 45 min in swine (Hunter, 1977).

Fertilization involves several steps occurring in a compulsory order. As oocytes arrive at the ampullary-isthmus junction, precapacitated spermatozoa contact oocyte at the zona pellucida. This contact is a relatively loose and nonspecific association, termed as attachment. Later, the association between the oocyte-sperm become relatively tenacious and species-specific, referred to as binding. Biologically, binding is mediated by sperm receptors present in the zona pellucida and specific oocyte-binding proteins residing in the sperm membrane.

The porcine zona contains 4 major proteins, zp1, zp2, zp3, and zp4 (Sacco et al., 1986). Immediately after the sperm-oocyte binding, spermatozoa undergo acrosomal reaction, penetrate the zona pellucida and fuse with oolemma. The fusion with the two gametes triggers a series of events to happen, including activation of the oocytes and blocking polyspermy. The cortical reaction involves fusion of plasma membrane and cortical granule membrane and deposition of cortical granule contents into perivitelline space. As a result, porcine sperm receptors are inactivated (Hedrick et al., 1987) and the oolemma is reorganized so that sperm can not attach to the inner zona pellucida nor fuse with the oolemma.

Coincident with the cortical reaction, the oocytes arrested in MII become activated. The chromosomes on the equatorial plate begin to move apart and spindle elongates.
Subsequently, the second polar body is formed. The remaining haploid maternal chromosomes disperse to form female pronucleus. As the zygotic chromosomes become decondensed, vesicles aggregate along the edge of dispersing chromosomes and fuse to form a bilaminar envelope (Longo, 1973). These processes result in the female chromosomes surrounded by two parallel membranes which subsequently fuse together forming an irregular shaped pronuclei (Longo, 1987).

As the female pronucleus is forming, the sperm head also undergoes rapid changes, such as breakdown of the nuclear envelope, dispersal of sperm chromatin and reconstitution of a pronuclear envelope (Longo, 1981). After the sperm nucleus is incorporated into the ooplasm, the envelope of sperm nucleus quickly breaks down. Sperm chromatin is directly exposed to the zygotic cytoplasm.

Once pronuclei have formed, both pronuclei immediately start RNA synthesis (Tesarik and Kopecny, 1989). DNA synthesis takes place several hours later (Longo, 1976; Tesarik and Kopecny, 1989). Pronuclei move from the peripheral to the center of the zygote. When the two pronuclei are closely apposed, their nuclear envelopes disintegrate (Austin, 1960). Thus, the whole process of fertilization includes all events between the association of the oocyte and sperm until late mitosis of the first cleavage. In swine, these take approximately 8 to 10 h (Hunter, 1972).

**Early Embryonic Development**

After the zygote stage, embryos enter into several mitotic divisions. The zygote, or 1-cell stage is quite large, having a low nuclear to cytoplasmic ratio. To attain a ratio similar
to somatic cells, cell divisions are without an increase in cell mass. This process is referred to as cleavage. Cleavage of the zygote is by vertical division through the main axes of the egg. The resulting daughter cells are called blastomeres. The plane of the second division is also vertical and passes through the main axes but at a right angle to the initial plane of cleavage, producing four blastomeres. The third cleavage division occurs approximately at a right angle to the second, producing eight blastomeres. This doubling sequence is carried on through the reminder of early cleavage. The initial cleavage division usually occurs simultaneously in all the blastomeres, but the synchronization is inevitably lost and blastomeres start dividing independently of each other. (Bazer et al., 1987). Cleavage, while still encased within the zona pellucida, is such that blastomeres have to accommodate themselves to this limited area. Once the embryo has formed 8 to 16 blastomeres, it is referred to as morula, due to its resemblance to a mulberry.

The objective of this dissertation is to test the effect of some steroids and growth factors and the interactions of follicular cells (theca, granulosa, and oocyte) on porcine oocyte maturation and the follicular cells proliferation and differentiation.
CHAPTER II

EFFECT OF OOCYTE ON PORCINE GRANULOSA AND CUMULUS CELLS

PROLIFERATION

ABSTRACT

Studies have previously demonstrated that the oocyte plays a role in granulosa cell differentiation. This study was designed to investigate whether porcine oocytes have a role in the proliferation of mural granulosa cells and cumulus cells. Cumulus cell-oocyte complexes (COC) and mural granulosa cells were collected from medium-sized follicles (3-6 mm in diameter) of porcine ovaries. To determine the role of the oocyte in granulosa cell proliferation, granulosa cells were equally seeded (1.0 x 10^5 live cells/ well) in 24- well plates as a monolayer for 48 h then treated with fresh medium (TCM-199), FSH (200ng/mL), or denuded oocyte-conditioned medium for 24 h. The same treatments were done in cultured cumulus cells for 12 h. The cultured cumulus cell-oocyte complexes (20 COC/well) also were treated with TCM-199 for 12 h. ^3H-thymidine (5μci/mL) was added for the last 6h of each treatment. Cell proliferation was quantified by determination of ^3H-thymidine incorporated into newly synthesized DNA. FSH and denuded oocyte -conditioned medium caused an increase in the proliferation of mural granulosa cells (30% and 42% enhancement,
respectively). Denuded oocyte-conditioned medium caused an increase (50%) in the proliferation of cumulus cells compared to the control cumulus cells. Denuded oocyte-conditioned medium enhanced the cumulus cell proliferation to a level close to that of intact complexes. FSH had no effect on proliferation in cumulus cells. These observations suggest that the oocyte may secrete one or more factors that can promote proliferation of cumulus and mural granulosa cells in pig.

INTRODUCTION

Toward the end of the oocyte’s growth phase, the granulosa cells differentiate into two subpopulations organized as pseudo stratified epithelia (Anderson et al., 1978): (1) mural granulosa cells attached to the basement membrane enclosing the follicle and (2) cumulus granulosa cells attached and metabolically coupled to the oocyte. In numerous studies, the growth, development, and function of granulosa cells have been examined by culturing the cells as a monolayer in the presence of various hormones and growth factors, but the interpretation of these studies is complicated because of the loss of association of these cells with the oocyte. It has been observed that cell division occurs more frequently in the population of granulosa cells nearest the oocyte than in the more distant cells (Hirshfield, 1986; Takaoka et al., 1985). An increasing body of evidence supports early observations that the oocyte may play a role in granulosa cell development and function. The oocyte can prevent precocious luteinization of granulosa cells since removal or death of the oocyte in situ appears to promote spontaneous luteinization and progesterone production (El-Fouly et al., 1970; Hubbard and Erickson 1988). Some studies suggest that mouse oocytes secrete
a specific, developmentally-regulated cumulus expansion-enabling factor that allows cumulus cells to undergo cumulus expansion in response to FSH (Buccione et al., 1990; Vanderhyden et al., 1990). By removing the oocyte from an oocyte-granulosa cell complex, Vanderhyden et al. (1990) showed that soluble factors secreted by mouse oocyte promote granulosa cell proliferation and help to maintain the structural organization of the follicle. This putative role of the oocyte in proliferation of granulosa cells is the subject of the present study.

**MATERIALS AND METHODS**

*Collection and Culture of Mural Granulosa Cells*

Mural granulosa cells were obtained from medium-sized (3-6 mm in diameter) antral follicles of the porcine ovaries. Porcine ovaries were collected from gilts less than 30 min after killing at a local abattoir. Ovaries were transferred to the laboratory in less than 30 min after collection. The ovaries were then soaked in 70% ethanol for approximately 1 minute and washed 10 times with double distilled water. Under aseptic conditions, granulosa cells were obtained as described (Chang, et al., 1993). Briefly, medium-sized follicles (3-6 mm in diameter) were aspirated with a 20-gauge needle and a 10-cc syringe. After collecting the cumulus cell-oocyte complexes, the fluid was centrifuged for 5 minutes at approximately 200 x g. The supernatant was removed and cells were resuspended by gentle mixing in TCM 199 (Gibco Laboratories, Grand Island, New York, USA), supplemented with 100 U/mL penicillin (Gibco Laboratories) and 100 µg/mL streptomycin (Gibco Laboratories), and recentrifuged. Removal of supernatant, resuspension, and centrifugation was repeated. After removing the remaining supernatant, the cells were suspended in TCM 199 supplemented
(TCM 199/S) with 10% fetal calf serum (FCS, Hyclone, Logan, UT). Cells were then equally seeded into 24-well Corning culture plates (#25820, Corning Glass Works, Corning, NY) at a density of \(1.0 \times 10^5\) live cells/well. The wells had been previously coated with 10% fetal calf serum by adding 1.0 mL TCM 199 plus 10% FCS to each well at least 2 hours prior to seeding. FCS coating was used to enhance cell attachment. Each well contained a total volume of 1.0 mL TCM 199/S. The cells were incubated for 48 hours (5% \(\text{CO}_2\), 95% air, 37°C). After this incubation period, cells were washed 3 times with TCM 199 then treated with fresh medium (TCM-199; control), FSH (200 ng/ mL), or denuded oocyte-conditioned medium. The cells were incubated for 24 h and \(^3\text{H}\)-thymidine (5 \(\mu\text{Ci} /\text{mL}\) was added for in the last 6 h to two third of the wells of each treatment and the other one third was used for protein assay.

**Collection and Culture of Oocyte-Cumulus Cell Complexes**

The porcine ovaries were transported in ambient temperature. Cumulus cell-oocyte complexes (COC) were collected from the follicular fluid aspirated from medium- sized antral follicle (3-6 mm in diameter). The complexes were then washed 3 times in fresh medium and divided into two groups, one group was kept as intact complexes and the other group was used to obtain separated cumulus cells from the oocytes (denuded oocyte). Denuded oocytes were prepared by removing the oocytes from the cumulus cell-oocyte complexes by repeated pipetting through a narrow glass pipette till the oocytes were completely separated from all cumulus cells. Denuded oocyte-conditioned medium was obtained by culturing denuded oocytes (20 oocytes/mL) for 48 h in TCM-199, then this medium was collected for cell treatment. Intact complexes (20 COC/mL) and oocyte-free cumulus cells (1.0x10^5 live
cells/well) were separately plated in 24-well plates that were previously coated with 10% FCS. After 48 h, the cultured cells were washed 3 times with fresh culture medium TCM 199. The intact complexes (COC) were treated with fresh culture medium TCM-199 and the oocyte-free cumulus cells were treated with fresh medium TCM-199 (control), FSH (200 ng/mL), or denuded oocyte-conditioned medium for 12 h. After 6 h treatment, $^3$H-thymidine (5μCi/mL) was added to four wells out of six in each row, and the complexes were incubated for an additional 6 h at 37°C.

**Thymidine incorporation assay**

Cell growth was analyzed by quantifying $^3$H-thymidine incorporation into newly synthesized DNA. The quantity of $^3$H-thymidine incorporated into DNA was determined as described previously by Hu et al., (1993). Briefly, following the removal of $^3$H-thymidine solution, cells were washed twice with ice-cold Ca++ and Mg++ free Hanks balance salt solution (HBSS). Cells were fixed with 3:1 methanol: acetic acid for 6-10 min. Thereafter, the cells were washed with 0.75 M trichloroacetic acid (0.75 M TCA) for 30 sec. To collect the incorporated nuclear $^3$H-thymidine, 500 μl of 0.2N NaOH was added to each well and shaken at 120 rpm for 4 h. For neutralization, 500 μl of 0.2 N HCl was added to each well. After 5 minutes mixing, aliquots (500 μl) of the alkaline hydroxylate solution were mixed with 5.0 mL of scintillation cocktail and the incorporated $^3$H-thymidine were determined in a Beckman LS 5801 scintillation counter.

**Protein assay**

Two out of 6 wells of each row of the treatment were taken for protein content estimation. Total protein content was chosen as an indicator of cell number at the end of each
culture period. The cell protein content in each well was determined using the Bio-Rad micro assay technique. Briefly, after removal of medium, cells were lysed and collected by adding 1 mL of 0.1 N NaOH (Sigma Chemical Co.) to each well. Aliquots of 160 µl from each well were mixed with 40µl Bio-Rad protein assay dye reagent concentrate (Bio-Rad laboratories, Richmond, CA). The absorbance was measured with a Beckman DU-70 spectrophotometer using 595 nm visible light.

Statistical analysis

All experiments were performed at least twice with different batches of ovaries and 2-3 replicates per batch. The results of each treatment were combined. Data are presented as mean ± standard deviation (S.D). In each experiment, data were evaluated with One-Way analysis of variance (ANOVA) followed by Duncan’s multiple range test (Duncan, 1955) to determine the statistical differences of the means of treatment groups to the mean of the control group. p values less than 0.05 are considered as statistically significant. All the statistical analyses were based on the ratio of radioactivity of the ³H-thymidine to protein content (µg) per well.

RESULTS

In Vitro Proliferation of Mural Granulosa Cells from Antral Follicles

To investigate a possible role for the oocyte in the regulation of proliferation of granulosa cells, mural granulosa cells were isolated from medium-sized (3-6 mm in diameter) antral follicles and cultured as a monolayer for 48 h then treated with fresh medium, FSH (200 ng/ mL), or medium conditioned by denuded oocytes for 24 h. Both FSH and
denuded oocyte-conditioned medium significantly \((p<0.05)\) stimulated the proliferation of porcine mural granulosa cells, as indicated by increasing the ratio of \(^3\)H-thymidine incorporation DPM per \(\mu\)g cell protein (increase about 30% and 42%, respectively) compared to the control; Figure 1).

In vitro growth of cumulus-oocyte complexes and oocyte free cumulus from antral follicles

To ascertain the role of the oocyte in the proliferation of cumulus granulosa cells, the incorporation of \(^3\)H-thymidine into newly synthesized DNA was determined. Oocyte-free cumulus cells (1.0x10^5 live cells/well) were separately plated in 24-well plates. The intact complexes (COC) were treated with fresh culture medium TCM-199 and the oocyte-free cumulus cells were treated with fresh medium TCM-199 (control), FSH (200 ng/ mL), or denuded oocyte-conditioned medium for 12 h. Denuded oocyte-conditioned medium caused a significant increase in the proliferation of cumulus cells (50 % enhancement) compared to the control cumulus cells (Figure 2). Denuded oocyte-conditioned medium enhanced the cumulus cell proliferation to a level close to that of intact complexes. FSH had no effect on the proliferation of cumulus cells.

DISCUSSION

That the oocyte may play a role in proliferation of the granulosa cell layers was suggested by earlier studies in rats wherein \(^3\)H-thymidine was infused \textit{in vivo} and labeling of the granulosa cells closest to the oocyte was greater than labeling of those closest to the
basement membrane (Hirshfield, 1986 and Takaoka et al., 1985). The results presented here clearly demonstrate that the porcine oocyte promotes the proliferation of granulosa cells and cumulus cells from antral follicles. First, cells cultured alone without oocyte or oocyte-conditioned medium resulted in a basal level of cell proliferation, and, second, medium conditioned by oocytes enhanced the cell proliferation level and restored it to level of the intact controls. In addition, medium conditioned by oocytes promoted granulosa cell proliferation of mural granulosa cells from antral follicles. Although the oocyte is normally coupled with its companion granulosa cells via gap junctions (Anderson and Albertini, 1976), the action of the oocyte on granulosa cell proliferation does not require contact of the oocyte with granulosa cells. The oocyte, therefore, stimulates granulosa cell proliferation by the production and secretion of not yet defined one or more soluble factors.

Mural granulosa cells cultured as monolayer in oocyte-conditioned medium showed an increase in \(^{3}\text{H}-\text{thymidine}\) incorporation compared to those cultured in unconditioned medium. The cells in vivo are relatively distant from the oocyte; they maintain a physical association with it only through the gap junctions that functionally link granulosa cells to one another and to the oocyte (Anderson and Albertini, 1976). However, the observation that an oocyte-secreted factor can stimulate the proliferation of mural granulosa cells suggests that the oocyte may be secreting a granulosa cell growth factor that could act on the mural granulosa cells, perhaps via the follicular fluid in a paracrine fashion.

That the mechanisms regulating follicular growth and differentiation are very complex is becoming increasingly clear as the potential participation of more and more factors in these processes is revealed. In addition to FSH, steroids (Goldenberg et al., 1972), theca cell-
produced factors (Bendell et al., 1988, and Skinner et al., 1987), and numerous other growth factors (Bendell et al., 1988, May et al., 1990, Skinner and Coffey, 1988) influence the proliferation of granulosa cells. Appreciation of the oocyte as a source of additional growth factors for granulosa cells suggests that studies using cultures of granulosa cells separated from oocytes may not precisely reflect the relative roles of the factors regulating proliferation and differentiation in vivo.

The oocyte-derived factor is not yet characterized. In our laboratory, Coskun et al. (1995) demonstrated that porcine oocytes secrete a heat-stable factor and this factor inhibits FSH-induced steroid production by mural granulosa cells. This results excludes most of the growth factors known to inhibit granulosa cell steroidogenesis such as epidermal growth factor (EGF) and Activin-A. The same study indicated that this factor(s) is a charcoal-extractable molecule(s); thus it may be a small molecule, possibly a steroid, but to our knowledge there are no reports showing that the oocyte is capable of producing any steroids. In the present study, the effect of FSH on granulosa cell proliferation was significantly similar to that of oocyte-conditioned medium but FSH did not have significant effect on cumulus cell proliferation. It is not known whether it is a commonly recognized factor, whether it is produced by other cell types, or whether its activity is specific to granulosa cells.
Figure 1: Effect of FSH and oocyte-conditioned medium on proliferation of mural granulosa cells. Mural granulosa cells were seeded (1.0 x 10^5 live cells/well) as a monolayer culture and treated with fresh medium (TCM-199; control), FSH (200 ng/mL), or denuded oocyte-conditioned medium (1 mL/well). Each bar represents the mean ±S.D. Both FSH and denuded oocyte-conditioned medium were able to significantly (p<0.05) stimulate the proliferation of porcine mural granulosa cells, as indicated by increasing the ratio of 3H-thymidine incorporation DPM per μg cell protein (increase about 30% and 42%, respectively) compared to the control.
$^3$H-Thymidine incorporation (DPM/6h/μg cell protein)

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N = 6

Figure 1
Figure 2: Incorporation of $^{3}H$-thymidine by cumulus cell-oocyte complexes, cultured in fresh medium (COC), and oocyte-free cumulus cells treated with fresh medium TCM-199 (CTL; control), FSH (200 ng/mL), or denuded oocyte-conditioned medium (OCM; 1 mL/well). Each bar represents the mean ±S.D. Denuded oocyte-conditioned medium were able to significantly ($p<0.05$) stimulate the proliferation of cumulus cells, as indicated by increasing the ratio of $^{3}H$-thymidine incorporation DPM per μg cell protein (increase about 50%) compared to the control. In cumulus cell-oocyte complex group (COC), the cell protein (μg) included oocytes and cumulus cell protein combined.
Figure 2

$^{3}$H-Thymidine incorporation/ DPM/ 6h/ µg cell protein

- CTL
- FSH
- OCM

- COC

N = 6
CHAPTER III

EFFECT OF CELL-TO-CELL INTERACTION ON PORCINE OOCYTE MATURATION AND GRANULOSA-THECAL CELL DIFFERENTIATION AND PROLIFERATION

ABSTRACT

Mesenchymal-derived thecal cells and epithelial-derived granulosa cells interaction in the porcine ovarian follicle was investigated. Experiments were designed to examine the proliferation and steroidogenesis of these cell types and the effects of these interactions on oocyte maturation.

In the first experiment, $^3$H-thymidine incorporation in theca and granulosa cells was increased approximately two-fold when incubated as co-cultures. Progesterone production by granulosa cells co-cultured with theca cells was reduced to 40% of that of granulosa cells cultured alone. On the other hand, androstenedione production by theca cells co-cultured with granulosa cells increased approximately 245% compared to that of cultured theca cells alone. In the second and third experiments, the effect of granulosa-theca cell communication on oocyte maturation was investigated. Porcine cumulus-oocyte complexes were incubated with granulosa cells cultured alone or with theca cells. When cultured with granulosa cells
alone, 30% of oocytes showed germinal vesicle breakdown after 10 hours of incubation. However, when incubated with co-cultured granulosa and theca cells, the same amount of germinal vesicle breakdown (30%) was significantly delayed to 24 hours. FSH induced a delay in germinal vesicle breakdown when complexes were incubated with co-cultured granulosa and theca cells. FSH did not exert this inhibitory effect when oocytes were cultured with granulosa cells alone. These results demonstrate that communication between these two somatic cell types within the ovarian follicle results in reciprocal modulation of their growth and function. Oocyte maturation can also be modified by the communication between these cells. It is concluded that a signal(s) produced by the interaction between granulosa and theca cells is essential in the maintenance of the original structure and function of the porcine follicular walls, as well as in modulating oocyte maturation under the effect of gonadotropins.

INTRODUCTION

Interactions between cells of mesenchymal and epithelial origin occur in essentially every organ and are among the most common types of cell-cell interaction involving different cell types. Mesenchymal cells are considered to be essential for epithelial proliferation, morphogenesis, and differentiation, especially in organs with a rapidly renewing epithelium, such as intestine, and in organs that have cycles of functional activity, such as those of the female reproductive system (reviewed by Donjacour and Cunha, 1991). It has long been recognized that the differentiation of epithelial cells is directed by adjacent mesenchymal cells during embryonic development and is optimally maintained by adjacent stroma in adult tissues (Kratochwil, 1972). Although the importance of mesenchymal-epithelial cell interactions has
been established for most tissues, the molecular mechanisms by which mesenchymal cells modulate epithelial structure and function are not well understood and remain to be elucidated.

The ovarian follicle is one of the most rapidly growing normal tissues. The proliferation of granulosa and theca cells, the two prominent somatic cell types in the follicle, is considered to be responsible for most of the follicular expansion that occurs during maturation. The mechanisms by which the growth and differentiation of granulosa and theca cells are controlled however is poorly characterized. During each reproductive cycle, the epithelial-derived granulosa cells support the oocyte and form the antrum of the follicle. The mesenchymal-derived thecal cells surround both the follicle and the outer layer of granulosa cells to provide structural integrity for the follicle. In addition, products of one of these cell types, theca or granulosa, are known to affect the function and/or proliferation of the other cell type lying on the opposite side of a separating basement membrane. For example, the androgen produced by theca cells is utilized by granulosa cells as a substrate for estrogen production (Dorrington et al., 1975), while progestins from granulosa cells serve as precursors for theca cell production of androstenedione (Fortune, 1986). Furthermore, it has recently become clear that a number of growth factors synthesized by ovarian cells have paracrine and autocrine actions at the level of granulosa and theca cells. The effects of several growth factors, including epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), transforming growth factors-α (TGF-α), transforming growth factors-β (TGF-β), keratinocyte growth factor (KGF/FGF-7) and hepatocyte growth factors (HGFs), on granulosa cells and theca internal cells have recently been reported by numerous investigators.
For example, while bovine theca cells express the gene for TGF-α and TGF-β, granulosa cells have the receptors for these factors (Skinner and Coffey, 1988; Lobb et al., 1989; Skinner et al., 1987). Bovine theca cells also produce a mitogenic factor with a molecular weight greater than 10,000 (Bendell et al., 1988). Recently, the proteins inhibin and activin, which are produced by granulosa cells and control FSH secretion from the anterior pituitary gland, have been purified from the follicular fluid of several species. These proteins are also known to exert paracrine effects, such as the regulation of theca cell androgen production (Bicsak and Hsueh, 1988). Therefore, mesenchymal-epithelial interactions between theca and granulosa cells appear to be important for ovarian physiology and follicle development. Co-culture of porcine oocytes with porcine granulosa cells inhibited the resumption of meiosis (Tsafirri and Channing, 1975; Coskun et al., 1995). This inhibitory action of granulosa cells depended on their concentration in vitro. Furthermore, extracts of granulosa cells and conditioned media from cultured granulosa cells were also shown to inhibit oocyte maturation (Tsafirri and Channing, 1976; Centola and Channing, 1981).

Co-culture systems have made it possible to explore the effects of intercellular communication on cellular morphology and function. Using this technology, the present study examined the interactions between porcine ovarian theca and granulosa cells.
MATERIALS AND METHODS

Experiment 1: Effect of granulosa-theca cell interactions on cellular proliferation and basal steroid hormone synthesis.

Preparation of porcine ovarian granulosa and theca cells

Porcine ovaries were collected from gilts less than 30 min post mortem at a local abattoir. Ovaries were transferred to the laboratory in less than 30 min after collection. The ovaries were then soaked in 70% ethanol for approximately 1 min and washed 10 times with double distilled water. Under aseptic conditions, granulosa cells were obtained as described by Chang et al., 1993). Briefly, medium-sized follicles (3-6 mm in diameter) were aspirated with a 20-gauge needle (Becton Dickinson & Co., Rutherford, NJ) and a 10-cc syringe (Becton Dickinson & Co.). After collecting the cumulus cell-oocyte complexes, granulosa cells were collected by centrifugation of the follicular fluid for 5 minutes at approximately 200x g. The supernatant was discarded and cells were washed twice by centrifugation in tissue culture medium 199 (TCM 199, Gibco Laboratories, Grand Island, NY) supplemented with 100U/ml penicillin and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY). The cells were then suspended in TCM 199 supplemented (TCM 199/S) with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and then seeded into 75 cm² Corning culture flasks (Corning Glass Works, Corning, NY) (10 mL of cell suspension per flask) which had been previously coated with 10% FCS in TCM 199 for at least 2 hours prior to seeding. FCS coating was used to enhance cell attachment. The cells were then cultured for 48 hours (5% CO₂, 37 °C) until use in experiments.
To collect theca cells, theca internal layers were removed from the follicular walls of aspirated follicles with fine forceps and rinsed thoroughly with TCM 199 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. The theca cell layers were then minced and digested in 1.0% collagenase, 1.0% hyaluronidase and 0.01% DNase (Sigma, Chemical Company, St Louis, MO) in Hanks balanced salt solution, pH 7.4 (HBSS; for 40-60 min at 37°C with continuous stirring. Dispersed theca cells were washed three times with culture medium, and then cultured in FCS-coated flasks as described above for granulosa cells for 48 h.

The cells were removed from the flasks by treatment for 5 to 10 min with 0.1% trypsin (Gibco Laboratories, Grand Island, NY) in HBSS, washed 3 times and equally seeded at density of 1 x 10^6 live cell / well in 12-well plate.

**Culture of granulosa and theca cells**

Figure 3 shows a diagram of the co-culture systems used to study the interactions between granulosa and theca cells. This system employs dual chambers that share the same culture medium via a permeable membrane in the upper chamber. (Cell Culture Insert, Cyclospore Membrane; Falcon, Becton Dickinson Labware, Lincoln Park, NJ). Thus, communication between cell types in the upper and lower chambers occurs through soluble factors released by the cells. To determine the effect of theca-granulosa cell co-culture on granulosa cell proliferation and progesterone production (Figure 3 A), freshly prepared granulosa cells were plated onto 12-well culture plates (lower chamber) and purified theca cells were plated onto Transwell co-culture inserts (upper chamber) (1 x 10^5 live cells/chamber). To determine the effect of theca-granulosa cell co-culture on theca cell
proliferation and androstenedione production (Figure 3 B), theca and granulosa cells were cultured in the lower and upper chambers, respectively. As controls, theca and granulosa cells (1x10^5 live cells/well) were cultured alone on 12-well plates. All cells were cultured for 48 h (37°C, humidified atmosphere, 5% CO_2). After the culture period, media were collected for steroid hormone radioimmunoassay (RIA) and 3H-thymidine incorporation assays were performed.

**3H-Thymidine incorporation assay**

The effects of heterologous intercellular communication on granulosa and theca cell proliferation were evaluated by measurement of 3H-thymidine incorporation. After removal of media for RIA, the cells were washed with TCM 199 and 9 of the 12 wells in a treatment group were pulsed with 5.0 μCi 3H-thymidine (DuPont NEN, Boston, MA) for 6 hours (5% CO_2, 37°C). The rates of 3H-thymidine incorporation were determined as described by Hu et al (1993). Briefly, following the removal of 3H-thymidine solution, cells were washed twice with ice-cold Ca**+- and Mg**+-free HBSS. Cells were fixed with methanol: acetic acid (3:1, v/v) for 6-10 minutes and then washed with 0.75 M trichloroacetic acid for 30 seconds to separate the cytoplasmic membrane. To collect the incorporated nuclear 3H-thymidine, 0.2 N NaOH was added to the plates (500 μL/well) which were then shaken at 120 rpm for 4 h at room temperature. The solutions were neutralized with equal volumes of 0.2 N HCl. After 5 minutes of mixing, aliquots (500 μL) of the alkaline hydroxylate solution were mixed with 5.0 mL of scintillation cocktail and the radioactivities of the incorporated 3H-thymidine were determined in a Beckman LS 5801 scintillation counter. Results were expressed as the ratio of incorporated radioactivity (DPM) to total protein (μg) per well.
**Protein assay**

The cells in three of the 12 wells in a treatment group that were not used for $^3$H-thymidine incorporation were used for total protein content estimation. Total protein content was chosen as an indicator of cell number at the end of each culture period. The cell protein content in each well was determined using the Bio-Rad micro assay technique. Briefly, after removal of medium, cells were lysed by the addition of 1 mL of 0.1 N NaOH (Sigma Chemical Co., St Louis, MO) to each well. An aliquot of 160 µl from each well was mixed with 40µl Bio-Rad protein assay dye reagent concentrate (Bio-Rad laboratories, Richmond, CA). Absorbances were measured with a Beckman DU-70 spectrophotometer using 595 nm visible light. Protein levels were determined from a standard curve generated using bovine serum albumin.

**Steroid assay**

To detect the effect of intercellular communication on granulosa and theca cell function, steroid hormone production by these cells was measured. At the end of the culture period, culture media were collected from each well and kept frozen. Progesterone and androstenedione were determined by RIA as described by Gu et al. (1990). Briefly, culture medium samples were extracted with ether (1:7, medium:ether, v:v). Aqueous layers were snap-frozen in a methanol-dry ice mixture, and the organic layers were collected, dried and reconstituted in phosphate buffered saline containing gelatin. Aliquots of the extracts were incubated with progesterone antisera (Endocrine Sciences, Tarzana, CA) and [1,2,5,7,21-$^3$H(N)]-progesterone (DuPont, New England Nuclear, Boston, MA) or androstenedione antisera (Endocrine Sciences, Tarzana, CA) and [1$^3$H(N)] (DuPont, New England Nuclear,
Boston, MA). Free and antibody-bound steroids were separated with dextran-coated activated charcoal. Radioactivities of the bound fractions were measured in a liquid scintillation counter and steroid concentrations were calculated from a standard curve. Progesterone (P₄) levels were expressed as ng/µg cell protein and androstenedione levels as pg/µg cell protein.

Experiment 2: Effect of granulosa-theca cell interactions on the meiosis-arresting activity of granulosa cells on porcine oocytes.

In this experiment, the time course of germinal vesicle breakdown in oocytes cultured with granulosa cells, alone or together with theca cells, was assessed.

Preparation of granulosa and theca cells

Granulosa and theca cells were prepared as described above in experiment # 1.

Preparation of cumulus cell-oocyte complexes

Ovaries, used in collecting cumulus-oocyte complexes, were transported in the ambient temperature. The follicular contents were recovered by aspiration from 3 to 6 mm diameter follicles using a 10-ml syringe and a 20-gauge needle. Cumulus-oocyte complexes were collected from the follicular fluid. Complexes were used for the following studies within 30 min of removal from the follicles. Only oocytes with tightly attached cumulus cells were used for the study.

Culture of cumulus cell-oocyte complexes

Porcine COC were cultured under 3 different conditions for three different incubation times to determine the effect of theca-granulosa cell co-culture on the ability of granulosa
cells to arrest meiosis in oocytes. The COC were cultured in the presence of culture medium alone, granulosa cells alone and co-cultured granulosa and theca cells for 10, 20 and 24 hours.

The cultured granulosa cells, co-cultured granulosa and theca cells, and culture medium (control) were incubated for a period of 48 hours prior to the addition of the COC. Twenty COC were then placed in each well and cultured (37°C, humidified atmosphere, 5% CO₂) for 10, 20 or 24 hours. The cumulus cell-complexes were then collected and assessed for stage of maturation.

**Evaluation of oocyte maturation**

At the end of culture, oocytes were mechanically denuded by repeated pipetting of the cumulus cell-oocyte complexes using a glass pipette with a narrow tip. Oocytes were then fixed, stained and examined under light microscopy.

Staining of oocytes. To determine the stages and rates of maturation of the oocytes, the cells were stained with orcein as described below. A drop of medium containing denuded oocytes was pipetted onto a glass slide and covered with a coverslip. For fixation, the slides were placed in an acetic acid-methanol (1:3, v/v) solution for 48 h at room temperature. The oocytes were then stained with 1% aceto-orcein solution and examined by light microscopy.

**Evaluation of oocytes.** Oocytes were classified according to the description of Hunter and Polge (1966) as germinal vesicle (GV, having an intact nuclear membrane with the chromatin of a meiotically inactive cell), or germinal vesicle breakdown (GVBD, having undergone a disrupted nuclear membrane and the chromatin pattern of an oocyte resuming meiosis).
Experiment 3: Effect of FSH treatment on the meiosis-arresting activity of granulosa cells on porcine oocytes.

In this experiment, the effects of FSH treatment on the ability of granulosa cells to arrest oocyte maturation were examined. Porcine COC were cultured under four different conditions for three different incubation periods (10, 20, 24 hours). Oocytes were cultured in the presence of granulosa cells alone, granulosa cells plus FSH (200 ng/mL; USDA), co-cultured granulosa and theca cells, and co-cultured granulosa and theca cells plus FSH (200 ng/mL). As described in Experiment 2, the four different conditions were incubated first for 48 h, then twenty COC were placed in each well and cultured (37°C, humidified atmosphere, 5% CO₂) for 10, 20 or 24 hours. The cumulus cell-oocyte complexes were then collected and assessed for stage of maturation as described above in Experiment 2.

Statistical analysis

All experiments were performed at least three times with different batches of ovaries and 2-3 replicates per batch. Data were expressed as mean ± standard deviation (S.D). In each experiment, data were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett multiple comparisons to compare the difference between each treatment mean and the control mean. A $p$ value of less than 0.05 was considered statistically significant.
RESULTS

Experiment 1: Effect of granulosa-theca cell interactions on cellular proliferation and basal steroid hormone synthesis

Cellular Proliferation

The effect of granulosa-theca interactions on cellular proliferation was examined by measuring the incorporation of $^3$H-thymidine into DNA. At the end of culture, proliferation was compared between the control (granulosa or theca cells cultured alone) and experimental groups (co-cultured granulosa and theca cells). The results show that co-cultured granulosa cells and theca cells exhibited a significant (2-fold) increase in the $^3$H-thymidine incorporation ($3020 \pm 127$ and $3700 \pm 160$ DPM/µg cell protein/6 h, respectively) compared to those cells cultured alone ($1500 \pm 63$ and $2000 \pm 100$ DPM/µg cell protein/6 h, respectively) (Figures 4 and 5). These observations indicate the existence of a not-yet-defined signal(s) between theca and granulosa cells that modulates DNA synthesis.

Progestosterone and Androstenedione Production

Progestosterone, but not androstenedione, was detected in the medium of granulosa cells cultured alone, while androstenedione, but not progesterone, was detected in the medium of theca cells cultured alone. Moreover, the media of co-cultured cells contained both progesterone and androstenedione (data not shown). Progestosterone production by co-cultured granulosa cells over 24 hours was significantly reduced as compared to that produced by granulosa cells cultured alone ($17.33 \pm 0.40$ vs. $43.61 \pm 2.24$ ng/µg protein, respectively; Figure 6). In contrast, androstenedione production by the co-cultured theca cells over 24
hours was significantly greater than that of the control group (427 ± 27 vs. 174 ± 3 pg/µg protein, respectively; Figure 7).

**Experiment 2: Effect of granulosa-theca cell interactions on the meiosis-arresting activity of granulosa cells on porcine oocytes.**

When COC were cultured with granulosa cells in the presence or absence of theca cells, tight attachment between the granulosa and cumulus cells was observed within 1-2 h of incubation and was confirmed by gentle agitation (not shown).

When COC were incubated in culture medium (control) alone, culture medium with granulosa cells alone, or co-cultured granulosa and theca cells, 50%, 30%, or 3%, respectively, of the oocytes underwent germinal vesicle breakdown within 10 h (Figure 8). The percentage of oocytes containing a germinal vesicle stage (GV; do not get mature) after culture with granulosa cells alone was significantly higher than that of control oocytes at 10 hours, but not at 20 and 24 hours (Figure 8). When COC were incubated with co-cultured granulosa and theca cells, the percentages of oocytes containing germinal vesicles after all incubation periods were significantly higher than those in oocytes cultured with medium alone or granulosa cells alone (Figure 8). Thus, these observations indicate a significant delay in germinal vesicle breakdown (oocyte maturation) when COC were incubated with co-cultured granulosa and theca cells, and, at 10 hours, show an enhancement of the ability of granulosa cells to arrest meiosis when co-cultured with theca cells.
Experiment 3: Effect of FSH treatment on the meiosis-arresting activity of granulosa cells on porcine oocytes.

When COC were cultured with FSH-treated granulosa cells alone, the percentages of oocytes containing germinal vesicles at 10, 20, and 24 hours of incubation were not significantly different from those of oocytes incubated with untreated granulosa (Figure 9). In contrast, FSH induced a delay in germinal vesicle breakdown when COC were cultured for 24 hours in the presence of co-cultured granulosa and theca cells (Figure 10).

DISCUSSION

These results demonstrate that communication between the two somatic cell types within the ovarian follicle results in reciprocal modulation of proliferation and function. The fact that proliferation and steroidogenic function of co-cultured cells were modified indicate the existence of signal(s) or other factor(s) between theca and granulosa cells that modulate these processes. Such interaction between two types of follicular cells may play an important role in follicular development and atresia.

To identify an effect of intercellular communication on granulosa and theca cell function, steroid hormone levels in culture media were measured. Androstenedione production by theca cells was increased when co-cultured with granulosa cells. This observation may result from granulosa cell production of progestin precursors for androstenedione synthesis, or a stimulation of steroidogenic activity via another mechanism.. Furthermore, progesterone levels within the medium from co-cultured cells were significantly lower than those in the medium of the granulosa cells cultured alone. This finding may be
explained by the conversion of progesterone to androgens by the co-cultured theca cells, or a reduction in steroidogenic activity induced by the theca cells.

The occurrence of germinal vesicle breakdown in porcine cumulus cell-enclosed oocytes cultured with granulosa cells was delayed after 10 hours of incubation compared to that cultured without granulosa cells (control) (Figure 8). This finding supports that of Tsafriri and Channing (1975) who reported that granulosa cells possess meiosis-arresting activity. Further delay in germinal vesicle breakdown was observed when COC were incubated with co-cultured granulosa and theca cells indicating that theca-granulosa cell interaction may play an important role in the meiosis-arresting activity of granulosa cells.

Germinal vesicle breakdown was further delayed when COC were incubated with co-cultured granulosa and theca cells that were supplemented with FSH. This result can be concluded to indicate that FSH, as well as a signal(s) or factor(s) produced by theca cells, may act directly on the oocyte or cumulus cells resulting in inhibition of meiotic maturation. Another possibility is that FSH and the theca cell signal(s) act at the level of the granulosa cell to enhance its meiosis-arresting activity. Eppig et al. (1983) demonstrated that FSH has meiosis-arresting activity on mouse oocytes.

The mechanism by which theca cells modulate the meiosis-arresting activity of granulosa cells is not well characterized yet. Skinner and Osteen (1988) reported that granulosa cells cultured alone in vitro undergo luteinization. In Experiment 1, the proliferation and steroidogenesis of granulosa cells co-cultured with theca cells were compared with those of granulosa cells cultured alone. While proliferation of the granulosa cells increased two-fold, their ability to secrete progesterone was reduced to one-third of that
of cells cultured alone (Figure 4 and 6). These observations of modulatory role of theca cells on granulosa cell steroidogenesis may suggest a possible role for theca cells in the inhibition of luteinization of granulosa cells during folliculogenesis in vitro. Perhaps the inhibition of granulosa cell luteinization by theca-granulosa cell interaction results in maintenance of the meiosis-arresting activity of granulosa cells.

The results indicate that communication between theca and granulosa cells was not by contact, but via molecules presence in the medium. Therefore, the produced signal(s) or factor(s) is a small soluble molecule which can pass through the membrane between the two chambers of co-culture system. This soluble factor(s) appears not to depend on the gap junctions between the cells to be transferred. From this observation, we can speculate that co-culture theca and granulosa cells can produce a conditioned medium which carries a factor(s) which can inhibit luteinization of granulosa cells, control oocyte development and maturation, and modulate theca and granulosa cell proliferation and differentiation.

In conclusion, interaction between theca and granulosa cells may play an important role in the meiosis-arresting activity of granulosa cells. FSH enhances the meiosis-arresting activity of granulosa cells in the presence of the theca cells.
Figure 3: Shows a diagram of the co-culture systems used to study the interactions between porcine ovarian granulosa and theca cells. This system employs dual chambers that share the same culture medium via a permeable membrane in the upper chamber. To determine the effect of theca-granulosa cell co-culture on granulosa cell proliferation and progesterone production (A). To determine the effect of theca-granulosa cell co-culture on theca cell proliferation and androstenedione production (B).
Figure 3

A

- Medium Level
- Theca Cells
- Granulosa Cells

B

- Medium Level
- Granulosa Cells
- Theca Cells
Figure 4: Effect of granulosa-theca cell interactions on granulosa cell proliferation. Cell proliferation was analyzed by quantitating $^3$H-thymidine incorporation into newly synthesized DNA. Granulosa cells (G.C) were cultured alone in 12-well plates at a density of $1.0 \times 10^5$ live cell/well (control). While in co-culture system between granulosa and theca cells (G.C.+T.C.), granulosa cells were cultured in the bottom chamber and theca cells in the upper chamber, each at a density of $1.0 \times 10^5$ live cells/chamber. After 48 hours of culture, cells were pulsed with $^3$H-thymidine 5 μCi/well for 6 hours. Data are expressed as mean ± S.D calculated from 6 independent experiments. The asterisk indicates a significant difference ($p<0.05$) between the two means. G.C.= Granulosa cells cultured alone, G.C.+T.C. = Granulosa cells and theca cells co-cultured.
Figure 4

$^{3}$H-Thymidine incorporation (DPM/6h/µg cell protein)
Figure 5: Effect of granulosa-theca cell interactions on theca cell proliferation. Cell proliferation was analyzed by quantitating $^{3}$H-thymidine incorporation into newly synthesized DNA. Theca (T.C) were cultured alone in 12-well plates at a density of $1.0 \times 10^5$ live cell/well (control). While in co-culture system between theca cells and granulosa (T.C.+G.C.), theca cells were cultured in the bottom chamber and granulosa cells in the upper chamber, each at a density of $1.0 \times 10^5$ live cells/chamber. After 48 hours of culture, cells were pulsed with $^{3}$H-thymidine $5 \mu$Ci/well for 6 hours. Data are expressed as mean ± S.D calculated from 6 independent experiments. The asterisk indicates a significant difference ($p<0.05$) between the two means. T.C= theca cells cultured alone, T.C.+G.C = theca and granulosa cells co-cultured.
Figure 5

3H-Thymidine incorporation (DPM/6h/µg cell protein)
Figure 6: Effect of granulosa-theca cell interactions on progesterone production by granulosa cells. Granulosa cells were cultured in 12-well plates at a density of $1.0 \times 10^5$ live cells/well in TCM 199 (control). As co-culture with theca cells, granulosa cells were cultured in the bottom chamber and theca cells in the upper chamber at a density of $1.0 \times 10^5$ live cells/each chamber. Data are presented as mean ± S.D. calculated from 6 independent experiments. The asterisk indicates a significant difference ($p<0.05$) between the two means. G.C. = Granulosa cells cultured alone, G.C.+T.C. = Granulosa cells and theca cells co-cultured
Figure 6

Progesterone (ng/μg cell protein/24 h)

N = 9
G.C. + T.C.
Figure 7: Effect of granulosa-theca cell interactions on androstenedione production by theca cells. Theca cells were cultured in 12-well plates at a density of $1.0 \times 10^5$ live cells/well in TCM 199 (control). As co-culture with granulosa cells, theca cells were cultured in the bottom chamber and granulosa cells in the upper chamber at a density of $1.0 \times 10^5$ live cells/each chamber. Data are presented as mean ± S.D. calculated from 6 independent experiments. The asterisk indicates a significant difference ($p<0.05$) between the two means. T.C. = Theca cells cultured alone, T.C.+G.C = Theca and granulosa cells co-cultured
Figure 8: Effect of granulosa-theca cell co-culture on the meiosis-arresting ability of granulosa cells. Percentages of porcine oocytes at the germinal vesicle (GV) stage of development were quantitated after culture of cumulus-oocyte complexes with (□) fresh culture medium “Control”, (□) with granulosa cells alone “G.C alone”, and (■) with co-cultured granulosa and theca cells “G.C+T.C”. for 10, 20, and 24 hours. The data are presented as mean ±S.D. Asterisks indicate significant differences (p<0.05) from the control groups in each time period. n shows the total number of oocytes examined in each group of three experiments.
Percentage of oocytes at germinal vesicle stage

Control  
With G.C alone  
With G.C + T.C

Time (hours)  
10  20  24

Figure 8
Figure 9: Effect of FSH on the meiosis-arresting activity of porcine granulosa cells. Percentages of porcine oocytes at the germinal vesicle (GV) stage of development were quantitated after culture of cumulus-oocyte complexes with granulosa cells in the presence (■) or absence (⊗) of FSH (200ng/mL) for 10, 20 and 24 hours. The data are presented as mean ± S.D. FSH did not exert a significant effect on oocyte maturation. n shows the total number of oocytes examined in each group of three experiments.
Percentage of oocytes at germinal vesicle stage

- **without FSH**: Shaded bars
- **with FSH**: Solid bars

**Time (hours)**

- 10
- 20
- 24

**Data Points**

- **n = 98 100 98 102 96 100**

*Figure 9*
Figure 10: Effect of FSH on the meiosis-arresting activity of granulosa cells co-cultured with theca cells. Percentages of porcine oocytes at the germinal vesicle (GV) stage of development were quantitated after culture of cumulus-oocyte complexes with co-cultured granulosa and theca cells in the presence (■) or absence (☆) of FSH (200 ng/mL) for 10, 20 and 24 hours. The data are presented as mean ±S.D. The asterisk indicates a significant difference ($p<0.05$) between the two means in each time period. FSH induced a delay in germinal vesicle breakdown when complexes were incubated with co-cultures granulosa and theca cells for 24 h. n shows the total number of oocytes examined in each group of three experiments.
Figure 10

Percentage of oocytes at germinal vesicle stage

- Without FSH
- With FSH

Time (hours)

10 20 24

n = 100 105 99 102 105 101

*
CHAPTER IV

EFFECTS OF STEROIDS ON RESUMPTION OF MEIOTIC MATURATION OF PORCINE OOCYTE-CUMULUS CELL COMPLEXES AND GRANULOSA CELL PROLIFERATION IN VITRO

ABSTRACT

Determination of optimal conditions for the in vitro maturation of porcine oocytes will increase the availability of in vitro matured oocytes for use in studies of early embryonic development in the pig. This study was designed to examine the effects of combinations of progesterone (P₄), testosterone (T), dihydrotestosterone (DHT) and follicle-stimulating hormone (FSH) on porcine oocyte maturation. Porcine ovaries were obtained from a local abattoir. Oocytes were aspirated from follicles (3-6 mm in diameter) and matured for 48 h in (a) tissue culture medium (TCM) 199 as control; (b) TCM 199 supplemented with FSH (2.5 µg/ml) only or in combination with P₄ (1 µg/ml), T (1 µg/ml) or DHT (1 µg/ml). After 48 h of culture, germinal vesicle breakdown (GVBD) and metaphase II (MII) were used as end-point parameters of oocyte maturation. Granulosa cell proliferation based on ³H-thymidine incorporation was also assessed. Maturation rates of oocyte exposed to the combination of FSH and P₄ or FSH and DHT were 73% (from a total of 125 oocytes) and 67.2% (127 oocytes), respectively. Although FSH alone induced a maturation rate (45.1% to 47.1%, 120
to 130 oocytes) that was lower than that seen when combined with P₄ or DHT, the rate was higher than those observed with control medium (33.3% to 36.0%, 83 to 87 oocytes), P₄ alone (38.4%, 81 oocytes), T alone (36.0%, 85 oocytes), and DHT alone (36.0%, 80 oocytes). Thus, the presence of P₄ or DHT enhanced the effect of FSH on maturation. At the concentration used in these experiments, T had no effect on porcine oocyte maturation, either alone or in combination with FSH. Thus, in the presence of FSH, T and DHT had different effects on maturation. ³H-Thymidine incorporation by granulosa cells was significantly increased in cultures treated with FSH and DHT (2-fold), but not in those treated with P₄ or T. The results indicate that these hormones may have different roles in regulating the maturation of porcine oocytes during follicular development in vivo.

INTRODUCTION

The mammalian oocyte enters meiosis during fetal life and is thereafter arrested in the dictyate stage of early meiotic prophase. Thirty to forty eight hours before ovulation the preovulatory surge of gonadotropins triggers the resumption of the meiotic process (Tsafriri, Pomerantz, and Channing, 1976). The resumption of meiosis is morphologically identified by breakdown of the germinal vesicle (GVBD) and is accompanied by expansion of the compact layers of cumulus cells surrounding the oocyte.

If immature oocytes harvested at slaughter could be induced to undergo normal maturation in vitro, a much larger proportion of the total population of oocytes in the ovary could provide a useful source for embryo transplantation. However, the culture of oocytes within intact follicles provides the only guaranteed method at present of inducing full
maturation of both the nuclear and cytoplasmic components in vitro (Moor and Cahill, 1980). The results of in vitro maturation studies have demonstrated the requirement for hormonal supplements in conjunction with follicular cell support (cumulus) for both nuclear and cytoplasmic maturation of oocytes (Suss et al., 1988; Madison et al., 1992). The responses to hormonal supplements for optimal in vitro development of oocytes are variable and not well defined (Down, 1993). Clearly, the understanding of the process of oocyte maturation is lagging behind the biological technologies available to the oocytes (in vitro fertilization, transgenic animals, embryo splitting). The association of follicular steroids (progesterone and estradiol) and prolactin with oocyte maturity and fertilizability has been investigated in human reproduction with the goal of identifying a marker associated with high quality oocytes (Fishel et al., 1983; Botero et al., 1984).

In vitro studies using gonadotropins have shown that FSH is able to induce GVBD in the presence of various meiotic inhibitors and that its effect on murine oocyte maturation is at first inhibitory and later stimulatory (Downs et al., 1988). Although the addition of LH to culture media did not affect the completion of maturation, the fertilizability and normal development of extrafollicular oocytes was increased (Shalgi et al., 1979). The significance of steroids in the control of the meiotic maturation of mammalian oocytes, remains controversial. While some studies have demonstrated that steroids, such as estrogens and progesterone, enhance the completion of maturation changes in both the nucleus and cytoplasm of mammalian oocytes (Bae and Foote, 1975; McGaughey, 1977; Moor et al., 1980; Fukui et al., 1982), other studies have demonstrated inhibitory estrogenic (Richter and McGaughey, 1979; Rice and McGaughey, 1981; McGaughey, 1982; Racowsky, 1983)
actions on the maturation of liberated oocytes. Yet, other investigations have revealed no such regulatory capacities for either estradiol (Smith and Tenny, 1980) or progesterone (Shea et al. 1975; Smith and Tenny 1980). It has been reported that major perturbations to the normal profile of steroids secreted by ovine follicles during maturation result in gross abnormalities during fertilization in vivo (Moor et al., 1980). The nature of these fertilization abnormalities is correlated with both the type of steroid perturbation induced and the stage of maturation at which it is induced. Mattioli et al. (1988) reported that progesterone is crucial both for the maintenance of intracellular coupling between porcine oocyte and cumulus cells during maturation and for subsequent male pronuclear formation. Many investigators have also indicated that steroids play an important role in the synthesis of the presumed male pronucleus growth factor (MPGF) which appears in the oocyte cytoplasm during natural maturation in vivo (Soupant; human (1974), Thibault et al.; mammals (1975) and Moor et al.; sheep (1980)). In addition progesterone and androgens have intrafollicular effects on follicular growth and steroidogenesis. The presence of progesterone receptors, along with high levels of intrafollicular progesterone, suggest a role for this hormone in follicular development. Progesterone was shown to enhance hCG-stimulated growth of small antral follicles in rats, as well as hCG-induced estrogen synthesis (Richards and Bogovich, 1982). Progesterone was also shown to enhance gonadotropin-stimulated secretion of progesterone in vivo and in vitro in rat (Fanjul et al., 1983). Although androgens are associated with atresia, these hormones have a role in preovulatory follicular development. Androgens can augment FSH-induced aromatase activity in rat granulosa cells, thereby enhancing estrogen synthesis (Daniel and Armstrong, 1980). Androgen can also stimulate the
biosynthesis of progesterone in granulosa cells. Administration of androgen, either in vivo or in vitro, can further stimulate production of progesterone by gonadotropins (Leung et al., 1979).

_in vivo_, the oocyte is exposed to an ever-changing milieu of gonadotropins, steroids, growth factors, and other molecules, any or all of which may interact to regulate maturational changes that occur in the oocyte and its surrounding cumulus cells during the periovulatory period. Generally, follicular steroids have been accepted as important for the development of bovine oocytes in vitro (Stubbings et al., 1990; Sirotkin, 1992). However, to date, there is little information about possible interactive effects of steroids (estradiol, progesterone, testosterone, and dihydrotestosterone) and gonadotropins in the regulation of porcine oocyte maturation and cumulus expansion. In vitro studies of these complex interactions among gonadotropins and steroids in oocyte maturation may lead to improved techniques for in vitro maturation (IVM) and thus to increase fertilization and embryonic development.

The present study was undertaken to examine the effects of testosterone (T), dihydrotestosterone (DHT) and progesterone (P₄) in various combinations with FSH and low density lipoprotein (LDL) on the initiation of meiotic maturation in the pig in vitro. These factors were chosen for study based on their presence in the ovary, their effects on oocyte maturation and granulosa cell proliferation and the aforementioned controversial reports regarding their roles in ovarian function.
MATERIALS AND METHODS

Chemicals and Reagents

Tissue culture medium (TCM 199) was purchased from Gibco Laboratories (Grand Island, NY). Progesterone, testosterone, and dihydrotestosterone were purchased from Sigma Chemical Company (St. Louis, MO). Fetal calf serum (FCS) was obtained from Hyclone Laboratories, Inc. (Logan, UT). Ovine follicle-stimulating hormone (FSH) was purchased from Reheis Chemical Company, Division of Armour Pharmaceutical Company (Chicago, IL). Stock solutions of progesterone, dihydrotestosterone and testosterone (1 mg/mL) were prepared in absolute ethanol and stored at 4°C. A stock solution of FSH (2.5 mg/mL) was prepared in sterile distilled water and stored as aliquots at -20°C. The final concentration of ethanol in treatment solutions in contact with cultured cells never exceeded 1μL/mL. As controls, equivalent volumes of ethanol were added to culture medium which did not contain steroids.

EXPERIMENT 1

Oocyte Maturation

Porcine ovaries were collected from a local slaughterhouse (The Herman Falter Packing Co., Columbus, OH) and then transported to the laboratory at ambient temperature within 30 minutes. The contents of follicles (3 - 6 mm in diameter) were recovered by aspiration using a 10-ml syringe and a 20-gauge needle. The cumulus-oocyte complexes (COC) were separated from the follicular contents and washed 3 times in TCM-199. Only those oocytes possessing a compact cumulus mass and dark ooplasm were selected for use.
in the study. Oocytes were cultured for 48 h (37°C, 5% CO₂, 100% humidity) in (a) TCM-199 only, as control, (b) TCM-199 supplemented with FSH (2.5µg/ml) only or in combination with P₄ (1µg/ml), T (1µg/ml) or DHT (1µg/ml). After 48 h of culture, germinal vesical breakdown (GVBD) and metaphase II were used as final parameters of oocyte maturation.

**Staining of oocytes.** To determine the stages and rates of maturation of the oocytes, the cells were stained with orcein as described below. The cumulus cells were removed from oocytes mechanically by repeated aspiration with a small bore pipette. A drop of medium containing oocytes was pipetted onto a glass slide and covered with a coverslip. For fixation, the slides were placed in an acetic acid-methanol (1:3, v/v) solution for 48 h at room temperature. The oocytes were then stained with 1% aceto-orcein solution and examined by light microscopy.

**Evaluation of oocytes.** Oocytes were classified as germinal vesicle (an intact nuclear membrane with the chromatin of a meiotically inactive cell), metaphase I (MI) (a disrupted nuclear membrane and the chromatin pattern of an oocyte resuming meiosis) or metaphase II (MII) (a polar body present in addition to chromosomes).

**EXPERIMENT 2**

*Porcine granulosa cell culture*

Porcine ovaries obtained from a local slaughterhouse were transported on ice within 30 minutes to the laboratory. The ovaries were then soaked in 70% ethanol for approximately 1 minute and washed three times with double distilled water. Granulosa cells were obtained from medium-sized follicles (3-6 mm in diameter) by aspiration with a 20
gauge needle and a 10 cc syringe (Becton Dickinson & Co., Rutherford, NJ). After removal of the COC, granulosa cells were collected by centrifugation of the follicular fluid (approximately 200 x g, 5 min). The granulosa cell pellet were washed three times by centrifugation in TCM-199. The cells were finally resuspended in TCM-199 supplemented (TCM-199/S) with 10% FCS (Hyclone, Logan, UT). Cells were then seeded into 24-well culture plates (#25820, Corning Glass Works, Corning, NY) at a density of 2.0 x 10^5 live cells/1.0 mL/well. The wells had been coated with 10% FCS in 1.0 mL TCM 199 at least 2 hours prior to seeding. FCS coating was used to enhance cell attachment. The cells were then cultured for 48 hours (5% CO₂, 37°C).

**Treatment of the cells**

After 48 h culture, the cells were washed three times with TCM 199 and then treated with \( P_4 \) (1.0 \( \mu \)g/mL), \( T \) (1.0\( \mu \)g/mL) and \( DHT \) (1.0\( \mu \)g/mL). After incubation for 24 h, cell proliferation was measured by the incorporation of \(^3\)H-thymidine. Each steroid treatment was applied to 6 replicate wells. The experiments were performed 3 times.

**\(^3\)H-Thymidine incorporation assay**

After 24 h treatment, the cells were washed with TCM 199 and pulsed with 5.0 \( \mu \)Ci \(^3\)H-thymidine for 6 h (5% CO₂, 37°C). The rates of H-thymidine incorporation were determined as described by Hu et al. (1993). Briefly, following the removal of \(^3\)H-thymidine solution, cells were washed twice with ice-cold Ca\(^{++}\) and Mg\(^{++}\)-free Hank's balanced salt solution (HBSS). Cells were fixed with methanol: acetic acid (3:1, v/v) for 6-10 minutes and washed with 0.75 M trichloroacetic acid for 30 seconds to separate the cytoplasmic
membrane. To collect the incorporated nuclear $^3$H-thymidine, 0.2 N NaOH was added to the plates (250 μL/well) which were then shaken at 120 rpm for 4 h at room temperature. The solutions were neutralized with an equal volumes of 0.2 N HCl. After 5 minutes of mixing, aliquots (500μl) of the alkaline hydroxylate solution were mixed with 5.0 mL of scintillation cocktail and the radioactivities of the incorporated $^3$H-thymidine were determined in a Beckman LS 5801 scintillation counter. Results were expressed as the ratio of radioactivity of incorporated $^3$H-thymidine (DPM) to total protein content (μg) per well.

**Protein assay**

The cells in 2 of the 6 wells that comprised a treatment group were used for total cell protein content estimation. Total protein content was chosen as an indicator of cell number at the end of each culture period. The cell protein content in each well was determined using the Bio-Rad micro assay technique. Briefly, after removal of medium, cells were lysed by the addition of 1 mL of 0.1 N NaOH (Sigma Chemical Co.) to each well. An aliquot of 160 μl from each well was mixed with 40 μl of Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Richmond, CA). Absorbances were measured with a Beckman DU-70 spectrophotometer using 595 nm visible light. Protein levels were determined from a standard curve generated using bovine serum albumin.

**Statistical analysis**

All experiments were performed at least three times with different batches of ovaries and 2-3 replicates per batch. Data were expressed as the mean ± standard deviation.
Differences among mean values of treatment groups were evaluated by analysis of variance (ANOVA) followed by Duncan’s multiple range test (Duncan, 1955). $P$ values of less than 0.05 were considered significant.

RESULTS

Treatment of COCs with steroids alone (P4 [1 µg/mL], DHT [1 µg/mL], or T [1 µg/mL]) did not alter oocyte maturation when compared to that observed for COCs cultured in control medium (unsupplemented TCM 199) (Figures 11, 12, 13). Furthermore, although treatment with FSH (2.5 µg/mL) elevated the mean percentages for oocyte maturation, the differences between these treatment groups and the controls were not statistically significant ($P<0.05$) (Figures 11, 12, 13). However, the combination of FSH with P4 (Figure 11) or DHT (Figure 12) significantly elevated oocyte maturation rate. Treatments that included T, however, had no effect on the proportion of oocytes that matured (Figure 13).

Incorporation of $^3$H-thymidine by cultured porcine granulosa cells was significantly increased by treatment with DHT (1µg/mL; 2-fold increase). No effect was induced by treatment with P4 or T at 1µg/mL concentrations. (Figure 14).

DISCUSSION

Mammalian oocytes have the unusual ability to undergo meiotic maturation spontaneously when released from follicles and cultured in vitro (Eppig, 1991). A variety of agents prevent spontaneous meiotic maturation in vitro at specific stages of nuclear progression. Germinal vesicle breakdown (GVBD) is the initial morphological feature
characteristic of meiotic maturation. The meiotic maturation represents the final stage of preparation of eggs for fertilization and early embryonic development. Accordingly, in this experiment, we choose the GVBD as a parameter of oocyte maturation affected by different supplementations and their combinations.

The study of oocyte maturation in mammals has followed two fundamentally different directions. In one, complex culture media containing serum or other heterogeneous biological fluids have been adopted for the investigation of the influence of various substances on oocytes. The second direction has been to employ a more clearly defined culture medium for oocyte maturation. The latter approach offers the advantage of examining the possible influence of specific components added to the medium on oocyte and granulosa cells, with a reduction in potential confounding interactions between the added components and constituents of the culture medium. This latter approach was employed for the present study to examine the effects of gonadotropin and steroid hormones as well as their combinations on porcine oocyte maturation and granulosa cell DNA synthesis.

A specific intrafollicular steroidal environment is required for the appropriate maturation of oocytes and for subsequent successful fertilization of bovine oocytes matured in vitro (Fukushima and Fukui, 1985; Zuelke and Brackett, 1990). Alteration of the micro environmental steroid profile during oocyte maturation results in gross abnormalities in both nuclear and cytoplasmic maturation of mammalian oocytes that lead to problems of fertilization (Moor et al., 1980; Osborn et al., 1986). The significance of follicular steroids for meiotic maturation of oocytes is quite variable among species (Racowsky and McGaughey, 1982). After the LH surge, when progesterone increases, nuclear maturation
and meiosis of bovine oocytes occurs (Grimes and Ireland, 1986). Wise et al. (1994) have shown that bovine oocytes with an intact germinal vesicle and compact cumulus layer have lower estradiol and progesterone than those exhibiting mature oocytes. In fact, a high concentration of estradiol in antral fluid has been used as a marker for human oocytes capable of undergoing fertilization (McNatty et al., 1979).

Observations that GVBD of bovine oocytes can be induced in vitro with gonadotropins in combination with steroids (Fukui et al., 1982) and blocked by inhibitors of steroidogenic enzymes in sheep (Moor, et al., 1980) indicate that the maturation-inducing substance is of ovarian origin and is a steroid. Although the effect of individual gonadotropins and steroids have been studied, there is a lack of data on the synergistic action of these two hormones on oocyte maturation in vitro. The results of the previous studies are conflicting. The reason for that may be because they viewed the possible role of steroids on oocyte maturation in vitro without combination with gonadotropins as in vivo. It is likely that these steroids interact with gonadotropins to regulate meiotic maturation of the oocyte.

The results of the present study demonstrate that porcine oocyte maturation in vitro is not significantly affected by FSH or steroids alone. This is in agreement with the findings of Hillensjo and Channing (1980) who reported that porcine oocyte maturation was not affected by FSH alone. However, Singh et al. (1993) reported contradictory results revealing that the addition of FSH (1.5 μg/mL) to TCM 199 medium increased the proportion of porcine oocytes undergoing GVBD, it noteworthy that the medium, they used, was supplemented with 25mM HEPES buffer, 15% fetal calf serum, 1mM L-glutamine, 0.03mM sodium pyruvate and 2.5 mM sodium lactate in addition that the incubations were carried out
at 39°C for 24 h which could be a reasonable reason of their different results. In addition, results of the present study indicate that, the presence of FSH and P₄ or DHT enhanced the resumption of meiosis in porcine oocytes in vitro, while T was without a significant effect (Figures 11, 12, 13). In pigs (McGauphy, 1977), rats (Richards and Bogovich, 1982), and cattle (Stubbings et al., 1990; Sirotkin, 1992), P₄ was shown to enhance oocyte maturation in vitro, while Fukui et al. (1982) reported no effect of the steroid on bovine oocyte maturation. Nevertheless, the enhancement of oocyte maturation observed in the presence of the combination of FSH and P₄ or DHT may be explained by the fact that FSH can induce and maintain receptors for P₄ and DHT in the follicle. This notion may be further supported by the fact that gonadotropins induce oocyte maturation by generating a positive maturational signal in the cumulus cells (Downs et al., 1988). Interestingly, DHT, but not T, significantly enhanced oocyte maturation and granulosa cell proliferation at the concentrations used in the present study (Figures 12, 13, 14). The reason for this observation is not clearly understood, but may be related to the fact that DHT is the most biologically potent form of androgen (Clark et al., 1992). The marked stimulation by DHT of ³H-thymidine incorporation in granulosa cells suggest that DHT may affect oocyte maturation indirectly via actions on the granulosa cells.

Much of the contradictory data describing the in vitro effects of steroids on oocyte maturation may be attributed to differences in species used, in the sizes/maturity of follicles collected, and in the stage of the estrous cycle at collection. In the present experiments, a chemically defined medium was utilized that did not include serum or albumin. Thus, the
methodology used for the study was appropriate for the efficient examination of the effects of single specific factors on the process of nuclear maturation in the porcine oocyte.

In conclusion, the finding that the addition of FSH to the culture medium is required for porcine oocytes to respond to steroids suggests that FSH may induce the production of steroid receptors in the COC. Furthermore, since the steroid-insensitive oocyte becomes steroid-sensitive (competent) in the presence of FSH in the medium, the development of maturational competence appears to be dependent on both gonadotropin and steroid hormones, suggesting an interaction between the peptide and steroid hormones in the process of oocyte maturation.
Figure 11: Effect of progesterone (P₄) and FSH alone and in combination on porcine oocyte maturation. Cumulus cell-oocyte complexes were cultured in unsupplemented medium (control) or medium containing FSH (2.5 µg/mL) in the presence or absence of P₄ (1µg/mL). After 48 hr, oocytes were assessed for GVBD as an indicator of maturation. The letter n indicates the total number of oocytes examined in each treatment group over 3 experiments. Data are presented as mean percentages ± S.D. calculated from 3 independent experiments. Asterisks indicate significant differences (P < 0.05) between P₄-treated and -untreated cells in the same treatment group.
Figure 11

Oocyte Maturation (%)

- White bar: Without P₄
- Black bar: With P₄ (1 µg/mL)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Without P₄</th>
<th>With P₄ (1 µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>FSH (2.5 µg/mL)</td>
<td>123</td>
<td>125</td>
</tr>
</tbody>
</table>

n = 87 81 123 125
Figure 12: Effect of dihydrotestosterone (DHT) and FSH alone or in combination on porcine oocyte maturation. Cumulus cell-oocyte complexes were cultured in unsupplemented medium (control) or medium containing FSH (2.5 µg/mL) in the presence or absence of DHT (1µg/mL). After 48 hr, oocytes were assessed for GVBD as an indicator of maturation. The letter n indicates the total number of oocytes examined in each treatment group over 3 experiments. Data are presented as mean percentages ± S.D. calculated from 3 independent experiments. Asterisks indicate significant differences ($P < 0.05$) between DHT-treated and -untreated cells in the same treatment group.
Figure 12

Oocyte Maturation (%)

- Without DHT
- With DHT (1μg/mL)

<table>
<thead>
<tr>
<th>Condition</th>
<th>None (n)</th>
<th>FSH (2.5 μg/mL) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without DHT</td>
<td>83</td>
<td>130</td>
</tr>
<tr>
<td>With DHT (1μg/mL)</td>
<td>80</td>
<td>127</td>
</tr>
</tbody>
</table>

*Significant difference
Figure 13: Effect of testosterone (T) and FSH alone or in combination on porcine oocyte maturation. Cumulus cell-oocyte complexes were cultured in unsupplemented medium (control) or medium containing FSH (2.5 µg/mL). After 48 hr, oocytes were assessed for GVBD as an indicator of maturation. The letter n indicates the total number of oocytes examined in each treatment group over 3 experiments. Data are presented as mean percentages ± S.D. calculated from 3 independent experiments. No significant difference was observed between T-treated and -untreated cells in the same treatment group.
Figure 13
Figure 14: Effect of steroids on porcine granulosa cell growth. Cell growth was analyzed by quantitating $^3$H-Thymidine incorporation into newly synthesized DNA. Cells were cultured in 24-well plates in TCM 199. After 48 h, cells were treated with no steroids (control) or one the steroids (1 μg/mL; $P_4$, T, and DHT). After 24 h treatment, $^3$H-Thymidine (5 μCi/ mL) was added to each well for the last 6 h. Data are presented as means ± S.D. calculated from 6 independent experiments. Asterisks indicate significant differences ($P<0.05$) compared to the control.
Figure 14

$^3$H-Thymidine incorporation (DPM/6 h/µg cell protein)

- Control
- P4 (1µg/mL)
- T (1µg/mL)
- DHT (1µg/mL)

N = 6
CHAPTER V

INFLUENCE OF KERATINOCYTE GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR-β1, ON IN VITRO MATURATION OF CUMULUS CELL-ENCLOSED PORCINE OOCYTE AND GRANULOSA CELL PROLIFERATION IN A DEFINED MEDIUM

ABSTRACT

The present study was undertaken to determine possible effects of various combinations of keratinocyte growth factor (hKGF), transforming growth factor-β1 (TGF-β1), follicle stimulating hormone (FSH), and luteinizing hormone (LH) on meiotic maturation and granulosa cell proliferation in the pig using an in vitro model system. Oocyte-cumulus cell complexes (OCC) were cultured in the media containing different combination of the above mentioned agents for 48 h and were observed for germinal vesicle breakdown (GVBD). Granulosa cell proliferation based on ³H-thymidine uptake was also assessed. The results showed that the treatment with hKGF significantly increased ($p<0.05$) incidence of GVBD in a dose dependent manner, with significant stimulation occurring at 10 ng/mL (63.2% vs 30% in the control) in oocyte maturation. Addition of h KGF (10 ng/mL) and FSH (2.5 μg/mL) together resulted in significantly higher ($p<0.05$) GVBD level than was observed in
hKGF treatment alone (85% vs 60%). $^3$H-thymidine incorporation by granulosa cells was significantly increased in cultures treated with 100 ng/ mL FSH (2.6-fold), 10 ng/ mL KGF (3.7-fold), and combination of FSH + KGF (5-fold) compared to the control. No significant effect was observed with transforming growth factor-β1 on GVBD, but TGFβ1 did exhibit a negative effect on granulosa cell growth. These results indicate that these growth factors may have different roles in oocyte maturation and that KGF has a beneficial effect on porcine oocyte maturation in vitro, suggesting a physiological role for KGF in vivo. In vitro effect of KGF may be indirect via paracrine stimulation of granulosa cells or induction of gonadotropin receptors resulting in enhanced oocyte maturation.

INTRODUCTION

The mammalian estrous cycle involves the cyclic processes of folliculogenesis, atresia, and ovulation. These processes necessitate coordinated differentiation and/or proliferation of the three major cell types within the follicle-granulosa cells, theca cells, and the oocyte (Hsueh et al., 1984). During each cycle, only selected follicle(s) ultimately mature to ovulation, whereas the majority regress through atresia. These events are partially under the endocrine regulation of gonadotropins, but a more precise mechanism must also function locally to regulate follicular development. The processes regulating the development and selection of ovulatory follicle(s) are presumably under endocrine as well as paracrine and/or autocrine control mechanisms. Since an epithelial-mesenchymal interaction exists between granulosa and theca cells, the theca cells have been postulated to modulate the effects of gonadotropins on granulosa cells through the production of specific factors (Skinner et al.,
It has become apparent, through recent research, that growth factors produced by theca and granulosa cells may locally modulate ovarian cell proliferation, differentiation, steroidogenesis, and angiogenesis (Hunter et al., 1992). In turn, factors that regulate granulosa cell function may alter communications between cumulus and oocyte and, thus, play important roles in regulating oocyte maturation. In addition, the factors, themselves, may act directly on the oocyte to influence its maturation.

Oocyte maturation has become an important subject of research since the advent of in vitro fertilization. Successful in vitro methods for oocyte maturation of farm animals could provide sufficient supply of oocytes for use in in vitro fertilization, basic research and biotechnology. Much of our understanding about the mechanisms controlling oocyte maturation is derived from in vitro oocyte studies. The potential physiological relevance of growth factors to granulosa and thecal cells has received significant attention during the last decade, however relatively few researchers have focused on the influence of growth factors on oocyte maturation. Now there is some evidence showing that oocyte growth and maturation may be regulated by an array of growth factors. This regulation may be under the control of central endocrine signals or, even in the absence of that central control, the resumption or arrest of meiosis may well be under paracrine or autocrine control of growth factors. Several growth factors have already been extensively reviewed (Ackland et al., 1992; Dye et al., 1992; Giudice, 1992).

Two of the identified mesenchymal derived growth factors that mediate mesenchymal-epithelial interactions are keratinocyte growth factor (KGF) and transforming growth factors-
β (TGF-β). KGF is a fibroblast growth factor (FGF-7)-related molecule that was originally isolated from keratinocyte-conditioned medium and found to stimulate epithelial cell proliferation (Rubin et al., 1989; Parrott et al., 1994). KGF is a 28-kilodalton protein that appears to be produced primarily by mesenchymal cells. KGF acts mainly as an epithelial cell mitogen (Finch et al., 1989). The receptor to KGF appears to primarily be localized on epithelial cells (Miki et al., 1992). TGF-β has been isolated in porcine ovary (Gangrade and May, 1990). TGF-β is an approximately 25 KDa peptide with homodimeric subunits and is produced by almost all mammalian cells investigated, normal and transformed (Hammond, 1981). The TGF-β family of peptide growth factors currently includes five members, TGF-β₁ to TGF-β₅. Our laboratory has previously demonstrated that TGF-β can regulate progesterone (P₄) production in gonadotropin-stimulated porcine granulosa cells in vitro (Chang et al., 1993a and b).

The present work was undertaken to evaluate the effect of KGF exogenously administered on in vitro porcine oocyte maturation and granulosa cell proliferation. These studies, as far as we know, have not been performed before on porcine or other domestic animals.

**MATERIALS AND METHODS**

**Oocyte Maturation**

Porcine ovaries were collected from a local slaughterhouse (The Herman Falter Packing Co., Columbus, OH) and were transported to the laboratory at ambient temperature
within 30 minutes. The follicular contents were recovered by aspiration from 3 to 6 mm
diameter follicles using a 10-ml syringe and a 20-gauge needle. The cumulus-oocyte
complexes (COC) were collected from the follicular contents and washed 3 times in tissue
culture medium 199 (TCM-199, Gibco Laboratories, Grand Island, NY). Only oocytes
possessing a compact cumulus mass and dark ooplasm were selected for the experiments.
Cumulus-oocytes complexes (20 to 30) were cultured in TCM 199 (2 mL), to which all
treatments were added, at 37°C in an atmosphere of 5% CO₂ and 100% relative humidity for
48 h. In this experiment, the effects of human recombinant KGF (hKGF, Pepro Tech Inc.,
Rocky Hill, NJ) and transforming growth factor-β₁ (TGF-β₁, R&D systems, Minneapolis,
MN) and their various combinations with FSH and LH (Reheis Chemical Company, Division
of Armour Pharmaceutical Company, Chicago, IL) on the initiation of meiotic maturation in
porcine oocyte in vitro were studied. The cultured COC were treated with (a) different
concentration of hKGF and TGF-β₁ (0, 1, 5, 10, and 25 ng/mL). (b) hKGF (10 ng/ mL)
only, or combined with FSH (2.5 µg/ mL) or LH (2.5 µg/ mL).

**Staining of oocytes.** To determine the stages and rates of maturation of the oocytes,
the cells were stained with orcein as described below. The cumulus cells were removed from
oocytes mechanically by repeated aspiration with a small bore pipette. A drop of medium
containing oocytes was pipetted onto a glass slide and covered with a coverslip. For fixation,
the slides were placed in an acetic acid-methanol (1:3, v/v) solution for 48 h at room
temperature. The oocytes were then stained with 1% aceto-orcein solution and examined by
light microscopy.

**Evaluation of oocytes.** Oocytes were classified according to the description of Hunter
and Polge (1966) as germinal vesicle (GV, having an intact nuclear membrane with the chromatin of a meiotically inactive cell), or germinal vesicle breakdown (GVBD, having undergone a disrupted nuclear membrane and the chromatin pattern of an oocyte resuming meiosis).

**Porcine granulosa cell culture**

Porcine ovaries obtained from a local slaughterhouse were transported on ice within 30 minutes to the laboratory. The ovaries were then soaked in 70% ethanol for approximately 1 minute and washed three times with double distilled water. Granulosa cells were obtained by aspirating medium-sized follicles (3-6 mm in diameter) with a 20 gauge needle and a 10 cc syringe. The COC were removed from the follicular fluid. The fluid was centrifuged for 5 minutes at approximately 200 x g. The supernatant was removed and cells were resuspended by gentle mixing in TCM 199 and recentrifuged. Removal of supernatant, resuspension, and centrifugation was repeated. After removing the remaining supernatant, the cells were suspended in TCM 199 supplemented (TCM 199/S) with 10% fetal calf serum (FCS, Hyclone, Logan, UT). Cells were then equally seeded into 24-well Corning culture plates (#25820, Corning Glass Works, Corning, NY) at a density of 2.0 x 10⁵ live cells/well. The wells had been previously coated with 10% fetal calf serum by adding 1.0 mL TCM199 plus 10% FCS to each well at least 2 hours prior to seeding. FCS coating was used to enhance cell attachment. Each well contained a total volume of 1.0 mL TCM 199/S. The cells were cultured for 48 hours (5% CO₂, 95% air, 37 °C).
Treatment of the cells

After 48 h culture, the cells were washed three times with fresh TCM 199 and treated with different concentrations of hKGF (0, 1, 5, and 10 ng/mL), TGF-β (0, 0.1, 1, 10 ng/mL), or hKGF (10 ng/mL) combined with FSH (100 ng/mL) or LH (100 ng/mL). The cells were incubated for 24 h and their growth was measured by determination of ³H-thymidine incorporation into newly synthesized DNA. The experiments were repeated 3 times.

³H-Thymidine incorporation assay

After 24 h treatment, the cells were washed with TCM 199 and pulsed (four wells out of 6) with 5.0 μCi/ml ³H-thymidine (DuPont NEN, Boston, MA.) in culture medium and incubated for further 6 hours (5% CO₂, 95% air, 37 °C). The rates of ³H-thymidine incorporation were determined as described by Hu et al. (1993). Briefly, following the removal of ³H-thymidine solution, cells were washed twice with ice-cold Ca++ and Mg++ free Hank's balance salt solution (HBSS). Cells were fixed with 3:1 methanol:acetic acid for 6-10 minutes. Thereafter, the cells were washed with 0.75 M trichloroacetic acid for 30 seconds. To collect the incorporated nuclear ³H-thymidine, 250 μl of 0.2 N NaOH was added to each well under constant shaking at 120 rpm for 4 h. For neutralization, 250 μl of 0.2 N HCL was added to each well. Then, aliquots (500μl) of the alkaline hydroxylate solution were mixed with 5.0 mL of scintillation cocktail and the incorporated ³H-thymidine were determined in a Beckman LS 5801 scintillation counter.
Protein assay

Two out of 6 wells of each row of the treatment were taken for protein content estimation. Total protein content was chosen as an indicator of cell number at the end of each culture period. The cell protein content in each well was determined using the Bio-Rad micro assay technique. Briefly, after removal of medium, cells were lysed and collected by adding 1 mL of 0.1 N NaOH (Sigma Chemical Co.) to each well. Aliquot of 160 μl from each well was mixed with 40μl Bio-Rad protein assay dye reagent concentrate (Bio-Rad laboratories, Richmond, CA). The absorbance was measured with a Beckman DU-70 spectrophotometer using 595 nm visible light.

Statistical analysis

All experiments were performed at least three times with different batches of ovaries. Data were expressed as the mean ± standard deviation. Differences in each group were evaluated by analysis of variance (ANOVA) followed by Dunnett multiple comparisons to compare the difference between each treatment mean and the control mean. A \( p \) value of less than 0.05 was considered significant. In the second experiment, all the statistical analyses were based on the ratio of radioactivity of the \(^{3}\)H-thymidine to protein content (μg) per well.

RESULTS

The actions of hKGF and TGF-β₁ on the porcine ovarian follicle were examined through an analysis of the mitogenic activity of the growth factors on oocyte maturation and granulosa cells.
EXPERIMENT No. 1

**Oocyte Maturation**

The effect of different concentrations (0, 1, 5, 10, and 25 ng/mL) of recombinant human KGF on COC is shown in Figure 15. In the control medium, 30 ± 5.3% of oocytes underwent germinal vesicle breakdown (GVBD). Germinal vesicle breakdown was enhanced in a dose-dependent manner, with 10 ng/mL hKGF being the most effective (63.2 ±3.9%). Therefore, 10 ng/mL hKGF was used in subsequent experiments to induce GVBD. Figure 16 shows the effect of 10 ng/mL hKGF alone or in combination with FSH (2.5 µg/mL) or LH (2.5 µg/mL) on the initiation of porcine oocyte maturation. Compared to the control medium, hKGF alone or in combination with gonadotropins did significantly increase the percentage of the porcine oocyte which underwent GVBD. Moreover, in comparison to the hKGF treatment alone, addition of FSH to hKGF treatment significantly increased the proportion of oocytes undergoing GVBD (85% vs 60%), while addition of LH to the hKGF treatment had no significant effect.

TGF-β₁ did not affect oocyte maturation when used at concentrations as high as 10 ng/mL. (Data not shown).

EXPERIMENT No. 2:

**Porcine granulosa cell culture**

After initial plating, freshly isolated granulosa cells were cultured in the absence or presence of different concentrations of hKGF (0, 1, 5, and 10 ng/mL) or TGF-β₁ (0, 0.1, 1, and 10 ng/mL) for 24 h. hKGF at 10 ng/mL was most effective at stimulating the
proliferation of porcine granulosa cells, as indicated by increases in thymidine incorporation (Figure 17). The hKGF effect was compared to that of FSH known to induce porcine granulosa cell proliferation. As shown in Figure 18, $^3$H-thymidine incorporation by granulosa cells was significantly increased in cultures treated with FSH (2.5-fold) or hKGF (3.7-fold) compared to the control, while addition of hKGF to the FSH-treated granulosa cells significantly ($P<0.05$) increased $^3$H-thymidine uptake by 5-fold compared to the control.

Figure 19 shows that all concentrations of TGF-β used in this experiment decreased $^3$H-thymidine incorporation. The thymidine uptake was reduced in a dose dependent manner, with 10 ng/ mL TGF-β being the most effective.

**DISCUSSION**

Growth factors are now being considered as potential regulators of ovarian function and follicular development. A complex local regulatory system under a central control must be present to selectively stimulate individual follicles towards final maturation which will eventually lead to resumption of meiosis.

Many studies on pig oocytes (Ding and Foxcroft, 1992, 1993 a, b; Zheng and Sirard, 1992; Nagai et al.,1993) showed significant enhancement of cytoplasmic maturation (indicated by the high male pronuclear formation rate) when cumulus-enclosed oocytes were cocultured with follicular shells or cultured in the follicular shell-condition medium or in the medium supplemented with follicular fluid. Moreover, piglets have been obtained from *in vitro* fertilized oocytes matured by cocultured with follicular shells (Mattioli et al., 1989) or in the medium supplemented with fractionated pig follicular fluid (Yoshida et al., 1993).
These results strongly indicate that follicular cells secrete factor(s) regulating nuclear and cytoplasmic maturation of the oocyte via paracrine and/or autocrine mechanisms. The precise nature of such stimulating factors in follicular secretions (follicular fluid and conditioned media) are not clear. Growth factors such as EGF (pig; Coskun and Lin, 1992, human; Das et al., 1991, bovine; Coskun et al., 1991; Harper and Brackett, 1993) and TGF-α (Brucker et al., 1991) have been demonstrated to stimulate or enhance nuclear maturation. These results suggest that growth factors may be one group of follicular factors involved in mediating both oocyte nuclear and cytoplasmic maturation. Previous observations have implied that theca cells may influence granulosa cell proliferation through the local production of TGF (Skinner et al., 1987; Skinner and Coffey, 1988). KGF has been implicated in ovarian follicle regulation and early embryonic development based on its production by theca cells (Parrott et al., 1994) and porcine endometrial stroma cells (Bartal et al., 1995). The present study has demonstrated that KGF is beneficial to porcine oocyte maturation. Furthermore, when combined with FSH, KGF was more effective than KGF alone in both enhancing oocyte maturation and stimulating marked increases in ³H-thymidine incorporation by granulosa cells. This enhancement by FSH of KGF-induced maturation may be due to the ability of FSH to induce and maintain KGF receptors in the follicle. The beneficial effect of KGF on granulosa cell proliferation seen in this study is consistent with recent bovine studies reporting a stimulation of granulosa cell proliferation (Parrott et al., 1994). In the present study, KGF in combination with FSH was more effective than KGF in combination with LH in enhancing porcine oocyte maturation, possibly due to the fact that cumulus cells have limited LH receptors (Channing et al., 1981). The same investigators found that bovine theca cells, but
not granulosa cells, express the KGF genes as well as synthesize and secrete the KGF protein.

Consistent with our observation, TGF-β did not significantly affect the maturation of mouse and rat oocyte (Downs, 1989; Tsafriri et al., 1989). Although TGF-β alone had no effect on spontaneous rat oocyte maturation, it suppressed LH-induced rat oocyte maturation in vitro (Tsafriri et al., 1989). Diverse effects of TGF-β between rats and pigs have been reported for progesterone production (Chang et al., 1993; Ohmura et al., 1993) and gonadotropin receptor regulation on granulosa cells (Gitay-Goren et al., 1993). TGF-β was detected in follicular fluid of small-sized porcine follicles (Gangrade and May, 1990) and found to inhibit FSH-stimulated progesterone production (Chang et al., 1993) and basal thymidine incorporation by (Mondschein et al., 1988) porcine granulosa cells, which is supported by our present results. These data suggest that TGF-β is involved in inhibiting differentiation of porcine granulosa cells, and possibly it is one of the factors responsible for follicular atresia. Although, in this study, TGF-β did not exhibit any stimulatory or inhibitory effect on maturation of oocytes from middle sized follicle, we cannot rule out its involvement in oocyte maturation in vivo. It may work on follicles of different sized or in combination with other factors in the follicle. Therefore, TGFβ1 was detected in follicular fluid of small-sized porcine follicle (Gangrade and May, 1990) and also it significantly reduced the thymidine uptake by granulosa cells in our present studies.

In conclusion, KGF augments porcine oocyte maturation, possibly by generation of a positive stimulus in cumulus cells which is transferred to the oocyte through gap junctions. This direct communication between cumulus cells and the oocyte via gap junctions plays an
indispensable role in the oocyte maturation process through the transfer of regulatory factor(s) in response to KGF. The high level of oocyte maturation achieved by supplementation of medium with a combination of hKGF and FSH, may be due to hKGF actions as an intraovarian regulator of oocyte maturation in response to gonadotropin stimulation.
Figure 15: Dose-response study of hKGF effect on GVBD. Oocyte-cumulus cell complexes were incubated in the medium containing varying concentrations of hKGF, and GVBD was assessed after 48 h. Data represent percent mean ± S.D. of three independent experiments. Asterisks indicate significant difference (p<0.05) compared to the control. Total number of oocytes examined (n) in each group in three replicates are indicated.
Figure 16: Effect of FSH (2.5 μg / mL), and LH (2.5 μg / mL) on KGF (10 ng / mL)-induced GVBD. Oocyte-cumulus cell complexes were cultured in the control medium and in medium containing hKGF or hKGF plus one of the above-mentioned hormones, and GVBD was assessed after 48 h. Data represent percent mean ± S.D. of three independent experiments. Asterisks indicate significant difference (p<0.05) compared to the hKGF group. Total number of oocytes examined (n) in each group in three experiments are indicated.
Figure 16

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Oocyte Maturation (%)
Figure 17: Effect of different concentrations of KGF on proliferation of granulosa cells as measured by $^3$H-thymidine incorporation into DNA. Data represent mean ± S.D. of the three independent experiments. Asterisks indicate significant difference ($P<0.05$) compared to the control.
Figure 17

$^3$H-Thymidine incorporation (DPM/6h/µg cell protein)

KGF (ng/mL)

N = 6

* Indicates statistical significance.
Figure 18: Effect of FSH (100 ng / mL) on recombinant human KGF (10 ng / mL)-induced granulosa cell proliferation. $^3$H-thymidine incorporation into DNA. Data represent mean ± S.D. of the three independent experiments. Asterisks indicate significant difference ($P<0.05$) compared to the control.
\(^3\)H-Thymidine incorporation (DPM/6h/\(\mu\)g cell protein)

N = 6

Figure 18
**Figure 19**: Effect of different concentrations of TGF-β1 on proliferation of granulosa cells as measured by $^3$H-thymidine incorporation into DNA. Data represent mean ± S.D. of the three independent experiments. Asterisks indicate significant difference ($P<0.05$) compared to the control.
CHAPTER VI

DEVELOPMENT OF PORCINE EMBRYOS GENERATED FROM IN VITRO-MATURED AND IN VITRO-FERTILIZED OOCYTES IN CHEMICALLY DEFINED MEDIUM

ABSTRACT

The development of in vitro maturation (IVM) and in vitro fertilization (IVF) techniques in pigs and in other species is of great importance because of possible applications of this technology in different research fields. Despite decades of use of porcine IVF and in vitro embryo development techniques, overall success rates remain low, and culture of porcine embryos to the blastocyst stage is at best only partially successful. Research efforts therefore have focused on the development and evaluation of modified IVM, IVF, and embryo development techniques. This study was designed to establish methodologies for future studies related to the early development of porcine embryos. Porcine follicular oocytes were matured in tissue culture medium 199 (TCM 199) as controls or in TCM 199 supplemented with 10 IU/ml hCG, 2.5 µg/ml FSH and 20 ng/ml PRL in the presence or absence of either 10% FCS or estradiol-17β (0.5 and 1.0 µg/ml). Germinal vesicle breakdown and metaphase II were used as final parameters for maturation. The five different
culture media resulted in maturation rates of 46.0%, 71.9%, 67.7, 86.8%, and 88.2%, respectively. Oocytes were then co-cultured with capacitated boar sperm for 6 to 8 h in IVF medium consisting of TCM 199 supplemented with 1 mM pyruvate, 3.05 mM glucose, 8.76 mM calcium lactate, 2 mM caffeine, 10% FCS and with or without 0.5 mM glycine. The fertilized ova were then cultured for an additional 120 h in TCM 199 supplemented with 40 ng/ml sodium pyruvate, 0.37% sodium lactate (60% w/w) and 10% FCS. A high rate of cleavage (52%) was achieved which resulted in normal embryonic development up to the morula or blastocyst stage. These results indicate that porcine oocytes matured in a hormonally supplemented medium can be fertilized and subsequently undergo cleavage and development in vitro up to morula and blastocyst stage embryos. Thus, valid techniques have been established to produce embryos for our future studies.

INTRODUCTION

The control mechanisms for mammalian oocyte meiotic maturation are one of the major puzzles of developmental biology and reproductive physiology. Oocyte maturation in vivo results from the combined effects of gonadotropins, originating from the endogenous preovulatory surge or exogenous administration, and steroids. In this respect, oocyte maturation is under the positive control of gonadotropins and steroids. However, when germinal vesicle stage oocytes or cumulus cell-oocyte complexes (COC) are removed from antral follicles and cultured, they undergo the nuclear events of oocyte maturation spontaneously (Ainsworth et al., 1980; Edwards, 1965; Eppig, 1991; Lenton et al., 1988). This observation led to the generally held conclusion that some component of the antral
follicle inhibits oocyte maturation until that inhibition is reversed as a result of the action of gonadotropins and steroids. The addition of hormone supplements to media enhances nuclear and cytoplasmic maturation, as well as cumulus expansion, of porcine COC (Funahashi and Day, 1993a; Prochazka et al., 1991; Yoshida et al., 1989). Thibault et al. (1975) and Moor et al. (1980) have indicated that steroids also play an important role in the synthesis of the presumptive male pronucleus growth factor (MPGF) which appears in the oocyte cytoplasm during natural maturation in vivo. It is still not certain, however, what role, if any, gonadotropins, estrogen, growth factors and other compounds have in the development of full maturational competence. The birth of live piglets resulting from the in vitro fertilization of oocytes that were matured in vivo has been reported only in the last decade (Cheng, 1985). Moreover, the ability of porcine oocytes to mature, be fertilized, and then develop in vitro, as well as the birth of piglets from in vitro matured and fertilized oocytes, has been demonstrated (Mattioli et al., 1989; Yoshida et al., 1990; Yoshida et al., 1993). While these studies demonstrated the development of viable 2- to 4-cell embryos from in vitro matured and fertilized oocytes, there has been to our knowledge no report of the in vitro development of viable blastocyst stage embryos from in vitro matured and fertilized oocytes. Factors which are predominant during late stages of follicular development in vivo may enhance the in vitro fertilizability and developmental capability of in vitro matured oocytes. The objectives of this study were to establish methodologies for future work related to the early development of porcine embryos and to investigate: (1) the direct maturation-promoting effects of gonadotropins and estradiol on porcine COC in vitro; (2) the in vitro fertilizability of oocytes matured in medium supplemented with gonadotropins and estradiol; and (3) the
developmental competence of porcine oocytes matured and fertilized *in vitro* and then cultured in hormonally defined embryo culture media.

**MATERIALS AND METHODS**

*Chemicals and Reagents*

Tissue culture medium (TCM 199) was purchased from Gibco Laboratories (Grand Island, NY, USA). Estradiol-17ß, human chorionic gonadotropin (hCG), sodium pyruvate, glycine, sodium lactate, Percoll and caffeine were purchased from Sigma Chemical Company (St. Louis, MO). Fetal calf serum (FCS) was obtained from Hyclone Laboratories, Inc. (Logan, UT). Ovine follicle stimulating hormone (oFSH) was purchased from Reheis Chemical Company, Division of Armour Pharmaceutical Company (Chicago, IL, USA). Ovine prolactin (oPRL) was provided by National Institute of Diabetes and Digestive and Kidney Diseases - National Institutes of Health (Bethesda, MD). Calcium lactate was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Glucose was purchased from Mallinckrodt, Inc. (St. Louis, MO).

*Oocyte Maturation*

Porcine ovaries were collected from a local slaughterhouse (The Herman Falter Packing Co., Columbus, OH) and were transported to the laboratory at ambient temperature within 2 h after death. The follicular contents were recovered by aspiration from 2 to 5 mm diameter follicles using a 5-ml syringe and a 20-gauge needle. The COC were collected from the follicular contents and washed 3 times in TCM-199. Only oocytes possessing a compact cumulus mass and dark ooplasm were selected for the experiments. Oocytes were cultured
for 48 h at 37°C in an atmosphere of 5% CO₂ and 100% humidity in 5 different culture media: (A) TCM-199 only as control, (B) TCM-199 supplemented with hCG (10 IU/ml), FSH (2.5 µg/ml) and PRL (20 ng/ml), C) TCM-199 supplemented with hCG (10 IU/ml), FSH (2.5 µg/ml), PRL (20 ng/ml) and 10% FCS, (D) TCM-199 supplemented with hCG (10 IU/ml), FSH (2.5 µg/ml), PRL (20 ng/ml) and estradiol-17β (0.5 µg/ml), or (E) TCM-199 supplemented with hCG (10 IU/ml), FSH (2.5 µg/ml), PRL (20 ng/ml) and estradiol-17β (1.0 µg/ml).

Staining of oocytes. To determine the stages and rates of maturation of the oocytes, the cells were stained with orcein as described below. The cumulus cells were removed from oocytes mechanically by repeated aspiration with a small bore pipette. A drop of medium containing oocytes was pipetted onto a glass slide and covered with a coverslip. For fixation, the slides were placed in an acetic acid-methanol (1:3, v/v) solution for 48 h at room temperature. The oocytes were then stained with 1% aceto-orcein solution and examined by light microscopy.

Evaluation of oocytes. Oocytes were classified as germinal vesicle (an intact nuclear membrane with the chromatin of a meiotically inactive cell), metaphase I (MI) (a disrupted nuclear membrane and the chromatin pattern of an oocyte resuming meiosis) or metaphase II (MII) (a polar body present in addition to chromosomes). Germinal vesicle breakdown (GVBD) (GVBD= MI+MII) and MII were used as final parameters to indicate oocyte maturation (Coskun and Lin, 1993). The oocytes remaining after staining were divided into groups (20 oocytes/group) and transferred to 2-ml of IVF medium containing aliquots of capacitated sperm cells (1 x 10⁶/ml). One group of each batch was recovered and incubated
in medium E for five days. The division rate of these porcine oocytes was used as a parameter for the rate of parthenogenesis.

**Sperm Capacitation**

Fresh porcine semen was purchased from Birchwood Genetic Inc. (West Manchester, OH.). Sperm capacitation was performed using the method described by Mattioli et al. (1989) with the following modifications: After the addition of glycine, caffeine and 10% FCS, and, after centrifugation, we collected the pellet of sperm that successfully penetrated the two layers (60 to 70%) of the Percoll gradient as opposed to that located between the layers (65 to 70%)

**In Vitro Fertilization**

Twenty oocytes with an expanded cumulus mass were placed into 35-mm culture dishes containing $1 \times 10^5$ sperm/ml in 2 ml of fertilization medium consisting of TCM-199 supplemented with 1mM pyruvate, 3.05 mM glucose, 8.76 mM calcium lactate, 2mM caffeine, 10% FCS and with or without 0.5 mM glycine. The spermatozoa and oocytes were incubated for 6-8 h at 39°C under an atmosphere of 5% CO$_2$ and 100% relative humidity.

**In Vitro Embryo Development**

The oocytes were removed from the surrounding cumulus cells and spermatozoa by using a narrow-bore glass pipette to reduce the incidence of polyspermy (Cheng, 1985). The oocytes were then cultured in embryo culture medium (TCM-199 supplemented with 40 mg
sodium pyruvate/L, 3.7 ml 60% sodium lactate/L, mixed antibiotics at 10 mg/L and 10% FCS). After 48 hours, the media were replaced to prevent the toxic accumulation of ammonium (Gardner et al., 1994). Seventy-two hours later, the oocytes were examined under phase-contrast microscopy and classified as uncleaved, 2-cell, 4-cell, and ≥8-cell stage.

**Statistical Analysis**

Two-sample T test and one-way analysis of variance followed by the Bonferroni Multiple Comparisons procedure were applied where appropriate. Differences between means were considered significant at $p < 0.05$.

**RESULTS**

A total of 1055 oocytes was used in the current studies. For each experimental group, at least ten trials were performed. The morphology of the COC population used was reasonably uniform and consistent (Plate 1).

Five different maturation media were compared for their abilities to produce mature oocytes. The compositions of the five media were as follows: medium A: TCM-199 only, medium B: TCM-199 with hCG (10 IU/ml), FSH (2.5 μg/ml), PRL (20 ng/ml), medium C: medium B with FCS (10%), medium D: medium B with estradiol-17β (0.5 μg/ml) and medium E: medium B with estradiol-17β (1.0 μg/ml).

To assess oocyte maturation, a subset of oocytes from each group was denuded and stained before the start of the embryo culture experiments. As shown in Figure 20, when COCs were cultured for 48 h, the percentages of oocytes that matured to germinal vesicle
breakdown (GVBD) and second metaphase (M II) were significantly higher in maturation medium E, (88.2% and 70.7%, respectively) than in media A, B, or C (46.15%, 27.0%; 72.0%, 41.5% and 67.7%, 36%, respectively). Oocyte maturation in medium E was significantly higher than that in medium D with respect to MII oocytes only (70.7% vs 54.0%, respectively).

To determine whether glycine has beneficial effects on sperm capacitation and embryo cleavage, the oocytes matured in medium E were fertilized in IVF medium with or without the addition of glycine (0.5 mM). As shown in Figure 21, the cleavage rate was significantly higher in IVF medium supplemented with glycine than in IVF medium without glycine. (67% ± 6.2 vs. 40% ± 5.4).

Since the maturation rate of oocytes was highest in those matured in supplemented medium E, the fertilizability and subsequent developmental competence of these oocytes was evaluated and compared to that of oocytes matured in media A and B. At 120 h after insemination, zygotes were evaluated to determine cleavage rates and stages of embryo development. As shown in Figure 22, the cleavage rates of embryos at various stages of development derived from oocytes matured in medium E were significantly higher than those of oocytes matured in media A and B (67.0%, 30.6% and 46.0%, respectively).

To evaluate the occurrence of parthenogenesis of porcine oocytes matured in defined simple medium (media), porcine oocytes were incubated in medium E. Medium E was selected for this test because it was the most effective one in inducing maturation then cleavage. Results show that only 2 to 5% of porcine oocytes developed beyond two-cell stage embryos.
The morphology of a typical developmental series of cultured porcine zygotes matured and fertilized \textit{in vitro} is displayed in Plates 5, 6.

\textbf{DISCUSSION}

At the present time, porcine oocytes matured and fertilized \textit{in vitro} cannot develop \textit{in vitro} beyond the 2- to 4-cell stage (2- to 4-cell block) (Herrmann and Holt, 1981). For this reason, all studies reporting the birth of piglets from \textit{in vitro} matured and fertilized oocytes involved the surgical transfer of 2- to 4-cell embryos into the oviducts of recipient pigs (Mattioli et al., 1989; Yoshida et al., 1990; Yoshida et al., 1993). On the other hand, successful \textit{in vitro} development of blastocyst stage embryos has been demonstrated using \textit{in vivo} matured and fertilized oocytes cultured in the presence of oviductal epithelial cells (White et al., 1989) or porcine oviductal fluid (Archibong et al., 1989), or in mouse oviduct maintained in organ culture (Krisher et al., 1989).

The methodology in the current study involved two distinct processes which must be considered separately: IVM and IVF/embryo development. The presence of cumulus cells in the COC is known to confer normal maturation and fertilization when gonadotropins and estradiol-17β are present in a static IVM system. Such information has been revealed by the work of investigators such as Nagai et al. (1993) who reported high rates of both maturation (M II) and male pronucleus formation when porcine COCs included $1.5 \times 10^4$ cumulus cells and that meiotic progression was significantly depressed when non-static culture systems were used for the maturation of COCs. Mattioli et al. (1988) have shown that the presence of a compact cumulus mass during maturation was necessary for the oocyte to undergo normal
fertilization and that, in a non-static system, porcine COCs matured in vitro showed low sperm penetration rates. Eppig (1981) demonstrated that the presence of cumulus cells during mouse oocyte maturation was important to achieve a high frequency of embryonic development. Bondioli and Wright (1983) incubated ram sperm with sheep cumulus cells to capacitate them prior to in vitro fertilization and subsequently obtained 2-cell embryos from ovarian oocytes, leading to their hypothesis that the cumulus cells aided in sperm capacitation. Coskun and Lin (1993) reported that EGF enhanced GVBD in porcine intact COCs while denuded oocytes were unaffected. Taken together, these data support our idea that COCs play a physiological role in the maturation and fertilization of porcine oocytes.

The beneficial effects of gonadotropins and estradiol-17β on maturation of porcine oocytes and cumulus expansion have been reported by many researchers whose findings agree with our results which indicate that the addition of gonadotropins into the maturation medium did not improve to any great extent the frequency of GVBD in the absence of estradiol. Thus, these findings suggest that gonadotropins alone were insufficient to provide a suitable environment for cytoplasmic and nuclear maturation of porcine oocytes. Moor and Trouson (1977) showed in sheep that the addition of estradiol to a medium containing FSH and LH resulted in the development of 26-50% of zygotes to blastocysts. In addition, disruption of the normal sequence of exposure to steroids during maturation of ovine oocytes resulted in clear intracellular aberrations during pronuclear development (Moor et al., 1980). Fukushima and Fukui (1985) showed that the addition of gonadotropins (FSH and LH) and steroids, especially estradiol, to culture medium is necessary to improve the fertilizability of extra follicular bovine oocytes matured in vitro. The presence of PRL, estradiol and gonadotropins
during *in vitro* maturation of rabbit oocytes was determined to be important for the promotion of preimplantation embryonic development (Yoshimura et al., 1989). Finally, Funahashi and Day (1993 a) reported a high rate of male pronucleus formation in porcine oocytes cultured for 20 h in medium containing PMSG, hCG and estradiol followed by 20 h culture in hormone-free medium. In the present study, the addition of estradiol to a medium containing FSH and hCG resulted in a significantly higher porcine oocyte maturation rate than medium containing the gonadotropins alone. Thus, both the steroid and the gonadotropins are required for physiological maturation and subsequent fertilization of porcine oocytes cultured under the present conditions.

Our results also showed that the rate of GVBD tended to decrease with the addition of fetal calf serum (FCS), (Figure 20). These results support those of Cran and Cheng (1986) who reported that the binding properties of FCS reduced the availability of calcium which is necessary for exocytosis and the cortical reaction. In addition, Naito et al. (1988) found that FCS inhibits maturation of porcine oocytes when added to modified Krebs-Ringer bicarbonate solution supplemented with FSH. Similarly, Funahashi and Day (1993 b) reported that supplementing maturation media with serum reduced the ability of porcine oocytes to form a male pronucleus.

The expansion of the bovine cumulus is significantly and positively correlated with the production of the glycosaminoglycan, hyaluronic acid, by the cumulus complex. Hyaluronic acid is highly effective at inducing acrosome reactions in bull spermatozoa *in vitro* (Handrow et al., 1982). Ball et al. (1983) showed that the *in vitro* induction of cumulus expansion by FSH prior to *in vitro* fertilization increased the incidence of bovine oocyte penetration and
pronucleus formation by bovine epididymal spermatozoa. Flechon et al. (1986) hypothesized that cumulus expansion proceeds from the superficial cell layers of the cumulus to the deeper ones, so that the corona layer is reached only at the end of maturation, thereby allowing the persistence of active intercellular coupling.

We modified the sperm capacitation technique and IVF medium described by Mattioli et al. (1989). These modifications resulted in a high level of fertilization and cleavage, suggesting that the treatment of sperm with Percoll gradient and the addition of caffeine and glycine to IVF medium were suitable for inducing capacitation. Capacitation involves the removal of epididymal or plasma components (coating proteins) from the sperm membrane, which permits the acrosome reaction to occur (Aonuma et al., 1973). Iritani et al. (1974) were the first to emphasize glycine as the predominant amino acid in oviductal fluid. Several investigators have used medium supplemented with 2mM caffeine for in vitro fertilization of porcine oocytes (Naito et al., 1988; Yoshida, 1987; Nagai et al., 1988; Hamano et al., 1989). It has been reported that caffeine enhances and prolongs the motility of bull and boar spermatozoa (Garbers et al., 1971; Garbers et al., 1973) and may accelerate the rate of sperm capacitation in mice (Fraser, 1979). Wang et al. (1991) reported that no sperm penetration occurred without caffeine, but that penetration was highest (89%) with 5mM caffeine in fertilization medium, while Mori et al. (1993) reported that addition of caffeine to IVF media resulted in significantly higher fertilization rates. Sperm separation on a discontinuous Percoll gradient with subsequent resuspension of spermatozoa in Ham's F-10 supports in vitro capacitation of equine spermatozoa (Arns and Shepherd, 1994). Our results of adding glycine support that results of Xia and Armstrong (1993) who reported that glycine is very important
for the porcine *in vitro* maturation, fertilization and early embryonic development.

The addition of pyruvate and lactate to embryo development culture medium results in good cleavage and embryo development. Pyruvate is a preferred substrate of preimplantation mouse (Leese and Barton, 1984) and human (Hardy et al., 1989) embryos. Furthermore, pyruvate as a sole energy substrate can support the development of a small proportion of hamster embryos to the blastocyst stage (Seshagiri and Bavisiter, 1989 a, b) and mouse embryos to the morula stage (Brown and Whittingham, 1991). Conaghan et al. (1993) showed that complete removal of pyruvate from the medium has a detrimental effect on the number of human embryos reaching the blastocyst stage. In a recent report of medium composition for the culture of bovine IVM/IVF oocytes, Kim et al. (1993) demonstrated that embryo development to the blastocyst stage can be achieved in simple chemically defined medium that includes amino acids, phosphate, pyruvate, lactate and, for development of later stages, glucose. Furthermore, the first cleavage was completely blocked in the absence of pyruvate and lactate, which they claimed are used as energy sources.

Currently, cytogenetic analyses are underway to determine whether blastocysts formed *in vitro* as described in this study are different from those resulting from natural mating. Nevertheless, in this study, the formation of blastocysts occurred at a rate similar to that observed *in vivo* (Hunter, 1974; Davis, 1985). Furthermore, since blastocyst formation *in vitro* is believed to be a valid indication of normal maturation, fertilization and development (Moor and Trouson, 1977), the present results provide evidence that suggests that the modified medium used in the present study can support the normal maturation and fertilization of porcine oocytes *in vitro*. Thus, valid techniques have been established to
produce embryos for future studies relating to early embryonic development, such as the effects of different growth factors on embryonic development and the subsequent viability of these embryos.

In conclusion, estradiol-17β is able to augment in vitro the spontaneous maturation of immature porcine oocytes which can subsequently undergo normal fertilization and cleavage (2- to >16-cell). The possibility of obtaining a large quantity of embryos capable of normal development represents an important step both for the study of gamete interaction and in future applications of biotechnology such as the introduction of foreign genes into early stage embryos and nuclear transplantation.
Figure 20: Effect of gonadotropins, estradiol and fetal calf serum (FCS) on *in vitro* porcine oocyte maturation. Porcine cumulus-oocyte complexes were cultured for 48 h in media of five different compositions (media A, B, C, D and E) and oocytes were then evaluated for degree of maturation after staining with orcein. PRL, FSH, hCG and FCS were present at 20 ng/ml, 2.5 µg/ml, 10 IU/ml and 10% (v/v), respectively. Values are expressed as means ± S.D. Among letters of the same case (upper and lower), different letters above bars indicate significant (*p* < 0.05) differences. GVBD = germinal vesicle breakdown; M II = metaphase II. *n* shows the average number of cumulus cell-oocyte complexes (COC) used in each experiment.
Figure 20

Oocyte Maturation (%)

<table>
<thead>
<tr>
<th>Medium</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRL+FSH+hCG</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FCS</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Estradiol /mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5μg</td>
<td>1.0μg</td>
</tr>
</tbody>
</table>

GVBD
MII
n = 120
Figure 21: Effect of glycine on cleavage rates of *in vitro* fertilized porcine oocytes. Porcine oocytes that were matured in medium E (see Fig. 20) were incubated for 6 - 8 h with capacitated boar sperm (10 COC with 1x10^5/ml) in IVF medium (see Materials and Methods for composition) in the presence or absence of glycine (0.5 mM). Cleavage rates were determined after examination of oocytes by phase-contrast microscopy. Values are expressed as means ± S.D. The asterisk (*) indicates a significant (p< 0.05) difference between the means. n shows the average number of cumulus cell-oocyte complexes used in each experiment.
Figure 21

Cleavage Rate (%)

Control

Glycine
(0.5 mM)

n = 118-139

*
Figure 22: Cleavage rates of *in vitro* fertilized porcine oocytes matured in different supplemented media. Porcine oocytes that were matured in medium A, B or E were fertilized in vitro (see Materials and Methods) and then evaluated 120 h after fertilization under phase-contrast microscopy to compare cleavage rates. Values are expressed as means ± S.D. Different letters above bars indicate significant (*p*< 0.05) differences among means. *n* shows the average number of matured oocyte used in each experiment.
Cleavage Rate (%)

<table>
<thead>
<tr>
<th>Medium</th>
<th>A</th>
<th>B</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH+PRL+hCG</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Estradiol (1μg/mL)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

n = 100

Figure 22
Plate I: Morphology of porcine cumulus-oocyte complexes (COC) after collected from an ovary, with dense cumulus cell layer, to be cultured in *in vitro* maturation media (IVM) for 48 h. Phase contrast micrograph (Mag. 200X).
Plate II: A porcine oocyte at germinal vesicle stage stained by 1% aceto-orceine. The nucleus staining is evidenced by a dark color (Mag. 800X).
Plate III: Metaphase I of a porcine oocyte. The nuclear membrane has been broken down and the chromosomes (Chr) can be seen clearly (Mag. 200X)
Plate III
Plate IV: A porcine oocyte at metaphase II. The first polar body (PB) is present and chromosomes (Chr) are identified in the cytoplasm (Meg. 800X).
Plate V: Morphology of cultured porcine zygotes matured and fertilized *in vitro* then cultured in embryo development medium for 48 h at 39°C in a humidified 5% CO₂ atmosphere. Phase-contrast micrographs (Mag. 200X) of 2-cell, 3-cell, and 4-cell embryo stages.
Plate VI: Morphology of cultured porcine zygotes matured and fertilized in vitro then cultured in embryo development medium for 120 h at 39°C in a humidified 5% CO₂ atmosphere. Phase-contrast micrographs (Mag. 200X) of 8-cell, compact morula, and blastocyst stages.
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