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Sanbuissho, Atsushi, Ph.D.

The Ohio State University, 1988
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UMI
INFLUENCE OF SERUM AND GONADOTROPINS ON IN VITRO BOVINE OOCYTE MATURATION AND FERTILIZATION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Atsushi Sanbuissho, D.V.M., M.S.

* * * * *

The Ohio State University
1988

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To my father
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PUBLICATIONS


FIELD OF STUDY

Major Field: Reproductive Physiology
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<td>BFF</td>
<td>bovine follicular fluid</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>ECS</td>
<td>estrous cow serum</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<td>FF</td>
<td>follicular fluid</td>
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<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>FSH-BI</td>
<td>follicle stimulating hormone binding inhibitor</td>
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<tr>
<td>GVBD</td>
<td>germinal vesicle breakdown</td>
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<td>HCG</td>
<td>human corionic gonadotropic hormone</td>
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<td>HTF</td>
<td>human tubal fluid</td>
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<td>LH</td>
<td>luteinizing hormone</td>
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<td>MPGF</td>
<td>male pronucleus growth factor</td>
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<td>OMI</td>
<td>oocyte maturation inhibitor</td>
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<td>polyacylamide gel electrophoresis</td>
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<td>SNDF</td>
<td>sperm nucleus decondensing factor</td>
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<td>SOF</td>
<td>synthetic oviductal fluid</td>
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CHAPTER I

INTRODUCTION

Several culture media have been used for in vitro culture including modified Tyrodes, Ham's F-10 with serum (Edward, 1981; Trounson et al., 1981), modified Whitten's medium (Trounson et al., 1982), Whittingams's T6 medium (Trounson et al., 1982) and Brackett medium (Brackett et al., 1982; 1984; Lambert et al., 1986).

The oocyte or embryo must derive its nutritional and environmental requirements from the biochemically complex medium and suitable atmosphere in an in vitro culture. One of the most important factors for successful in vitro fertilization is the selection of a suitable culture medium will may allow the ovum to undergo normal fertilization and embryonic development (Moor and Trounson, 1977; Trounson and Mohr, 1980). It is likely that the poor success rates in the past were at least partially due to the use of an inadequate culture medium.

As a protein supplement in medium, blood serum or its albumin fraction is an essential component of culture medium. The precise role of serum in supporting oocyte maturation and fertilization is not understood, but it is believed that
it provides protein and/or growth factors that contribute to the success of in vitro culture. Kane and Headon (1980) reported that the effect of serum albumin appeared to be due to the presence of a relatively high molecular weight protein.

Since the presence of serum macromolecules was found to be necessary for culture of oocytes, fetal calf serum (FCS; Shea et al., 1976; Choi et al., 1987), bovine serum albumin (BSA; Quinn et al., 1982; Kane, 1985), or maternal serum (Newcomb et al., 1978; Brackett et al., 1982; 1984) have been added to culture media. Fetal calf serum has often proved to be superior in promoting in vitro development of mammalian embryos (Wright et al., 1976). Presumably, the advantage of using fetal calf serum in a culture system arises because it contains some undefined growth promoting components that are absent from the serum of adult animals and/or that fetal serum lacks components (hormones and immunoglobulins) present in the adult serum that retard the in vitro development of cells.

When the blood serum and bovine serum albumin were compared as medium supplements in oocyte culture in vitro, a variation in the results was reported. Cross and Brinster (1970) reported that there was no significant difference between FCS and BSA in the number of oocytes completing maturation in mice. Leibfried et al. (1986) showed that the use of FCS was superior to BSA in promoting in vitro maturation and fertilization of bovine oocytes. Serum obtained from the cow during estrus has been used to
improve in vitro maturation (Newcomb et al., 1978) and fertilization rates (Lambert et al., 1986; Xu et al., 1987).

The role of gonadotropins in the in vitro maturation of the oocyte has also been acknowledged since resumption of meiosis was improved when gonadotropins were added to the medium (Thibault et al., 1975; Tsafriri and Channing, 1975; Thibault, 1977). Gonadotropins alter the metabolism of the cumulus cells and induce resumption of meiosis in the oocyte (Salustri and Siracuso, 1983) by interrupting a mode of inhibitory substances through the gap junction (Ball et al., 1983). In fact, maturation of the oocyte is induced by a specific stimulation of the preovulatory gonadotropic surge in vivo. The combined effect of FSH and LH on their target cells, and the metabolic interaction in the ovary must be considered (Eshkol et al., 1970).

The objectives of this study were to determine:

1. if there is an effect of bovine serum albumin (BSA), follicular fluid (FF), estrus cow serum (ECS), or fetal calf serum (FCS) as the medium supplement on the in vitro maturation of bovine follicular oocytes.

2. if there is an effect of BSA, FF, ECS, or FCS and their interaction with gonadotropins added to the medium on in vitro maturation of the bovine follicular oocyte.

3. if there is an effect of ECS or FCS and gonadotropins as a medium supplement on in vitro fertilization of the bovine follicular oocytes.
CHAPTER II

LITERATURE REVIEW

Composition of Culture Medium

Chang (1959) referred to the salt solution as acidic saline and supplemented it with glucose and serum in his initial rabbit in vitro fertilization experiments. Acidic saline supplemented with glucose and 5 or 10% heat treated rabbit serum was found to support in vitro fertilization (Brackett and Willeams, 1965). In addition to glucose for energy, simple fertilization medium supplemented with BSA, substituted for the protein content of serum was found to be adequate through earlier work. Brinster (1970) reported that BSA supported development of two-cell to morula stage rabbit embryos. The simple defined fertilization medium was found inadequate for zygote culture, thus ova were transferred into a serum supplemented medium following sperm penetration (Brackett, 1969).

The pH of the culture medium seems to be critical. Several different pH buffers have been used to avoid use of a CO2 atmosphere. Phosphate buffer, as might be expected, was useless for in vitro fertilization (Bavister, 1981). The bicarbonate CO2 buffer system did regulate the pH in
culture media. Fertilization occurs over a wide pH range (7.0-7.8) but the optimum is approximately 7.4. Bicarbonate and CO2 in equilibrium are most commonly employed to the buffer media and regulate pH in the culture medium. Serum enriched phosphate buffered saline with low bicarbonate content proved inferior for culture of viable sheep embryos compared to synthetic oviductal fluid, which is bicarbonate buffered (Tervit and Gould, 1978). In addition to its role in maintaining constant pH, bicarbonate is important in media to sustain embryo development, for blastocyst formation and for blastocyst expansion. High concentrations of bicarbonate are present in the rabbit oviductal and uterine fluid (Hammer and Willeams, 1964; David et al., 1969). Cross (1974) demonstrated that the amount of bicarbonate necessary for blastocyst expansion in the rabbit is greater than the bicarbonate produced metabolically by the embryo and therefore, most be supplied by the culture medium.

Whitten (1977) reported that 5% oxygen allowed the development of one-cell mouse embryos into blastocysts, whereas no blastocysts developed in 0 or 20% oxygen. Excess oxygen may result in oxidative damage to the embryo. Bovine embryos have been found to develop better under 5% oxygen than under 20% oxygen (Wright et al., 1976; Betterbed and Wright, 1985) but a significant difference in the development of ovine embryos was not found under these two
oxygen percentages (Wright et al., 1976). Brinster and Troike (1979) concluded that various levels of oxygen possibly influence development by altering the oxidation reduction potential in the ovum and/or embryos.

A wide range in osmotic pressure seems to be acceptable for in vitro culture. The hamster and mouse oocytes can be fertilized in the culture medium with osmotic pressures of 177-496 and 250-388 mOsM, respectively (Miyamoto and Chang, 1973). There is no sharply defined osmotic pressure optimum demonstrated for in vitro fertilization in any mammalian species. Development of in vitro fertilized rabbit zygotes to the eight-cell stage can occur at 221 mOsM (Brackett, 1969). A simple chemically defined medium with an osmolarity of 242 mOsM supported development of mouse zygotes to the blastocyst (Whitten and Biggers, 1968). Slightly hypotonic conditions may favor early embryonic development in vitro. The optimal osmolarity for development of two-cell mouse embryos was found to be 276 mOsM (Brinster, 1965).

The ratios of the principal ionic concentrations and the concentrations of chloride and bicarbonate are spread over a wide range but the majority of the successful culture media fall within a narrow range. The oocytes or embryo culture in these media may simply reflect the tolerance to the conditions; the gametes of some other species may not be able to tolerate the narrow range of ionic composition.
This difference could at least partly explain the difficulty in culturing oocytes of some species in vitro. Ions are necessary for successful development including Na+, K+, Ca++, Mg++, Cl−, P04−−, and HCO3−. Optimum levels of potassium, calcium, magnesium and phosphate are similar to serum values (Wales, 1970).

Fajer (1983) suggested that the changes in glycolytic metabolism observed in meiotic ovaries were attributed to the germ cells at the onset of meiosis. The permeability of the germ cells to glucose, lactate, and pyruvate has not been studied in many species. Experiments have shown that the early mouse embryos accumulate glucose and have a small intracellular pool of pyruvate (Wales, 1975). The integrity of the junctional contact between the oocyte and granulosa cells is required for the uptake of 2-deoxyglucose by the oocyte (Brower and Schultz, 1982). In the beginning, the oocyte needs only pyruvate in the culture medium but the two-cell stage is able to develop when supplied with pyruvate, oxaloacetate and lactate. Addition of lactate and pyruvate increases the incidence of spermatozoa undergoing an acrosome reaction (Bavister and Yanagimachi, 1977). During the first 2 days of development in the mouse embryos, 90-100% of the oxygen consumption of the embryo can be attributed to pyruvate oxidation (Brinster, 1967). Glucose oxidation is very low, accounting for less than 10% of the oxygen consumption by the mouse ovum but the oxidation
increases 100-fold during the preimplantation period with major increases at fertilization and during blastocyst formation (Brinster, 1967).

Protein is an important component of the culture medium. BSA was used as a protein supplement in most earlier work. It has been suggested that the supplementation of amino acids results in stabilization of the membranes and reduction in leakage of endogenous amino acids from the embryos (Brinster, 1971) or as a means for adding unidentified nutrients to the medium (Kane, 1979). The role of a commercial BSA preparation on rabbit zygote development to the blastocyst in vitro was thought due to the energy provided by albumin bound fatty acid and that blastocyst hatching was promoted by a non albumin component (Kane and Headon, 1980).

Although fertilization of cow ova can take place in vitro in a simple defined medium, more complex media are necessary for cleavage (Brackett et al., 1980). Wright et al. (1976) found that Ham's F-10 (Ham, 1963) supplemented with 10% heat treated fetal calf serum in 5% CO2, 5% O2, 90% N2 atmosphere progressed the viability of the cow embryo from the one-cell to the early blastocyst stage. As is the case for in vivo fertilized zygotes, in vitro fertilized embryos apparently require additional, as yet unknown, factors that ensure their continued development after in vitro culture (Seidel et al., 1976). The contribution of
the female reproductive tract supplies specific embryonic requirements as indicated from studies in the rabbit (Kendle and Telford, 1970). In addition to the hormonal influence, a role for changes in proteins within the female luminal fluids (Hall et al., 1978) upon zygote development must also be defined. Much is known of the composition of oviductal and uterine fluid under various hormonal condition and in different sites of the tubular female reproductive tract. It is possible that oviductal factors are necessary for normal fertilization and preimplantation development in vivo (Kapur and Johnson, 1986). Oliphant et al. (1984) have shown that the rabbit glycoproteins produced by the oviductal epithelium function as inhibitors of complement-mediated cell lysis. This inhibiting activity might be required to prevent maternal rejection of the embryo but might not influence in vitro fertilization and development. Some success in prolonged culture of sheep and cow embryos has followed efforts to duplicate the known composition of sheep oviductal fluid (Restall and Wales, 1966) with a synthetic oviductal fluid medium (Tervit and Gould, 1978).

**Culture Medium Supplement**

Culture media are usually supplemented with serum since protein supplementation improves embryonic development compared with serum-free medium (Saito et al., 1984).
Following sperm-egg fusion, a variety of metabolic pathways are activated concomitant with increased protein and nucleic acid synthesis (Wolf, 1982). The increased amount of protein may have some protective effects on the embryo or the protein growth factors may promote embryonic development (Johnstone et al., 1983). The beneficial components of serum are primarily in the large-molecular-weight fractions rather than the small molecules (Shirley et al., 1985). Bovine embryos may require a macromolecular component be present in the culture media (Allen et al., 1982). Non albumin components of sera may be responsible for this development. In contrast, Boone et al (1978) and Canfield et al (1986) suggested that BSA alone is capable of sustaining early bovine embryo development. Albumins pass into the uterus from the blood (Roberts and Parker, 1974) and may play a direct role in maintaining a suitable environment for embryos. It is reported that the presence of serum in culture medium during maturation in vitro can improve further development of fertilized eggs (Schroeder and Eppig, 1984). Choi et al. (1987) demonstrated that the incidence of sperm penetration of the oocyte increased with the increase in the concentration of fetal calf serum. De Felici and Siracusa (1982) reported that increased resistance of the zona pellucida to proteolytic dissolution, referred to as zona hardening, occurred during the maturation of oocytes in a chemically defined medium. The presence of
fetal calf serum in the medium influences spontaneous zona pellucida hardening as well as the incidence of sperm penetration. De Felici et al. (1985) have reported that follicular fluid contains factors that prevent spontaneous zona pellucida hardening during in-vitro maturation of mouse oocytes. These factors could possibly be derived from serum.

Metabolic substances are necessary for the growth of the oocyte (Heller et al., 1981; Pitts and Bark, 1976; Jagiello et al, 1977). Since cumulus cells are coupled with one another, nutrients in the blood of the dam can be transferred to the oocyte through gap junctions (Heller and schultz, 1980). These metabolic substances provided to the oocyte are known to play an important part in oocyte protein synthesis. The complex physiological and biochemical processes and structural changes are initiated in the nuclear and cytoplasmic components of the oocyte. A variety of metabolic molecules, such as amino acids, phosphorylilitic sugars, and nucleotides are transferred to the oocyte (Gilula, 1977). The mature oocyte contains about twice as much protein as the immature oocyte and albumin is taken up by the oocyte throughout its growth phase (Schultz et al., 1979). It has been suggested that protein synthesis undergoes dramatic qualitative changes during the process of oocyte maturation although a constant rate of protein synthesis is observed throughout the process (Kangia et al., 1977). A protein normally synthesized in the natural
process of oocyte maturation may be related to fertilization and further successful development. The absence of protein synthesis in the oocyte might be responsible for the failure of normal cleavage (Moor and Trounson, 1977).

Attempted fertilization of immature oocytes which have not completed preovulatory meiotic maturation results in a reduced fertilizability reflected by a premature release of cortical granules (Rosseau et al., 1977). After culturing, the adherent cells spread out over the surrounding surface and in some cases, the presence of the first polar body can be visualized. Immature human oocytes have been matured and fertilized in vitro (Thibault, 1977; Soupart and Morgenstern 1973; Veeck et al., 1983) and upon transfer have resulted in pregnancy (Veeck et al., 1983). The culture system which was used to mature bovine oocytes in vitro and which, after in vivo fertilization and retransfer to a recipient resulted in the birth of two calves, utilized Ham’s F-10 medium which was supplemented with estrous cow serum, 10 ug/ml estradiol and 1 IU HCG/ml (Newcomb, Christie and Rowson, 1978). Xu et al. (1987) and Xu and Greve (1988) also reported that a pregnancy obtained from oocytes matured and fertilized in vitro using estrous cow serum as medium supplement.

The follicular fluid is a liquid that accumulates in extracellular spaces within the follicle. The follicular fluid originates from two sources, plasma and the cells of the follicles. The concentration of sodium and potassium in
follicular fluid and in serum are different in cows. However, the other ions measured (magnesium, chloride, calcium, zinc, copper and inorganic phosphate) are found in similar concentration in the follicular fluid and the serum. The total protein concentrations in the fluid is either similar to or lower than that in serum. Both protein (LH, FSH, and Prolactin) and steroid hormones (estrogen and progesterone) are found in follicular fluid. LH and FSH levels increase in larger follicles, whereas prolactin concentrations in the follicular fluid is high in small follicles and low in large follicles. The concentration of steroids changes throughout follicular growth. Spicer and Zinn (1987) found that the concentration of cortisol in the fluid of small follicles was greater than in large follicles. Glucocorticoids have been shown to inhibit FSH-induced aromatase activity and stimulate FSH-induced progesterone production in rat granulosa cells (Schoonmaker and Erickson, 1983). Thus, greater concentrations of cortisol and progesterone and lower concentrations of estradiol are present in small the follicle.

The other follicular component which is important for oocyte physiology is the oocyte maturation inhibitor (OMI) released from granulosa cells (Tsafriri and Channing, 1975). Meiotic arrest of oocytes in antral follicles is mediated by the OMI found in porcine follicles (Tsafriri et al., 1976). The OMI from porcine follicles also maintains meiotic
arrest in mouse (Stone et al., 1978) and rat oocytes (Tsafriri and Channing, 1975) cultured in vitro. OMI-like activity is shown by bovine and hamster follicular fluid on hamster oocytes (Gwastkin and Anderson, 1976). However, it has been shown that follicular fluid was inefficient in preventing resumption of meiosis in the cow (Sreenan, 1970). Leibfried and First (1980) also showed that neither bovine nor porcine follicular fluid inhibited the spontaneous maturation of bovine oocytes in vitro.

Oviductal fluid provides a suitable environment for the fertilization and cleavage of fertilized ovum (Eyestone et al., 1987). Early embryonic development in the oviduct may be due to the presence of some specific compounds of oviductal origin and may alter of the oviductal environment which permits normal embryo growth (Bavister, 1988). This fluid is the product of the secretory activity of the endosalpinx (the secretory epithelium of the oviduct) as well as transudation from blood (Brackett and Mastroianni, 1974). Many oviductal fluid proteins are identical to those of serum; however, several are specific to oviductal secretion (Beier, 1974). Even though the oviduct is permeable to most serum proteins, there is a selective transport of some serum proteins into the oviductal lumen and the presence of unique proteins would seem to reflect the secretory activity of oviductal cells (Oliphant et al., 1984). Olds and VanDemark (1957) have collected oviductal fluids from
the cow continuously by catheter during the estrous cycle. The mean secretion of the oviductal fluid (ml/24 hours) were estrus, 5.31±1.57; metestrus, 3.60±0.55; diestrus, 1.49±0.11; proestrus, 1.36±0.34. The volume was larger during estrogen stimulation. However, there was no significant difference in protein concentration present during the different stages of the estrous cycle. The oviductal fluid collected from ewes throughout the estrous cycle is similar to that of the cow. As measured after cannulation of the oviduct, the fluid volume is greatest around estrus and diminishes during the luteal phase of the cycle. Albumin and transferrin appear present in relatively high concentrations compared with those in serum, but oviductal fluid lacks macroglobulin and some prominent fractions in the post-transferrin and immunoglobulin region (Beier, 1974). A number of proteins specific to oviductal fluid have been described and several of these proteins are produced only at particular stages of the estrous cycle (Mastroianni, L. Jr. and K. J. Go, 1979). In the sheep, there is an estrous-associated glycoprotein in oviductal fluid which is present for 3 to 6 days of the estrous cycle appearing on day 0 (Sutton et al., 1984; Sutton, Nancarrow and Wallace, 1986). Shapim et al (1974) showed that oviductal proteins form a high molecular weight aggregate with other components to make up the mucin coat of early rabbit embryos. The mucin coat is not clearly visible for
the embryo of other species; however, it is possible that oviductal proteins in these species bind to the ovum and facilitate fertilization and early embryonic development.

The ionic composition of oviductal fluid in human is characterized by high concentration of K and Cl, but low concentration of Ca relative to corresponding serum levels (Borland et al., 1980). The calcium and chloride levels of cow oviductal fluid are approximately the same as those of blood. In the ewe, the lowest levels of sodium and bicarbonate are present during the metestrous stage. Human tubal fluid contains 4 to 5 fold higher concentration of K than serum (David et al., 1973). In the mouse, significantly more embryos developed to fully expanded blastocyst in human tubal fluid (HTF) medium than in Tyrode's (T6) medium (Quinn, Kerin and Warnes, 1985). The result showed that there were liner responses in the number of embryos developing to expanded blastocyst with increasing levels of K. The Ca concentration in oviductal fluid is less than in serum, reflecting a lower concentration of protein in oviductal fluid than in serum (David et al., 1973). A total of 20 free amino acids in bovine oviductal fluid were found by Stanke et al. (1974). Glycine, glutamic acid, alanine, lysine and arginine are present in highest amounts. In human in vitro fertilization, three times as many pregnancies occurred when HTF medium was used for fertilization and culture compared with T6 medium
Terwit et al (1972) formulated a culture medium (synthetic oviduct fluid, SOF) similar to the biochemical composition of sheep oviductal fluid and obtained a significant improvement in the cleavage rates of sheep and cow embryos (Quinn et. al., 1984). The culture conditions formulated were as close as possible to those found in the natural environment. The oviduct produces a considerable amount of fluid through transudation and secretion (David et al., 1969). The protein profiles from oviductal fluid of the rabbit and rhesus monkey indicate that specific proteins are formed by the oviductal epithelium (Marcus and Saravis, 1965). Many mammals, including the cow, have oviductal epithelium that produces secretory granules during estrus and which are extruded into the lumen after ovulation (Brower and Anderson, 1969). The oviduct regulates the protein environment during fertilization and early development (Henault and Black, 1987). Rabbit embryos in the pronuclear stage were incubated in three days post ovulation oviductal fluid, all divided and 60% of them reached maturity. However, pronuclei oocytes did not divide when incubated in estrous rabbit oviductal fluid (Hamner, 1970). This indicates that the oviductal fluid has a special composition for at least the first three days after ovulation. It may be different from the fluid during other hormonal stages and therefore, support early cleavage and
development of the embryo and nutrients may be delivered to the oviductal environment as a result of endocrine and embryonic influences on the oviduct.

**Inhibition of Meiotic Resumption**

Oocytes remain in arrested meiotic prophase of nuclear maturation in the ovary. Pincus and Enzymann (1935) first reported this phenomenon and suggested that the maturation of the follicular oocyte is prevented by a substance produced by follicular cells which inhibits nuclear maturation. The most evident changes in oocyte structure during the final process of meiotic maturation is characterized by germinal vesicle breakdown (GVBD), formation and emission of the first polar body, and arrest at metaphase II (Albertini, 1984). Since oocytes in the ovary are at the germinal vesicle stage prior to the preovulatory surge of gonadotropins (Eppig, 1983), the endogeneous gonadotropic surge or the administration of exogeneous gonadotropins to a prepared female animal results in meiotic maturation of the oocyte (Eppig, 1984).

An inhibitory effect of follicular fluid upon the spontaneous maturation of isolated rabbit oocytes was first described by Chang (1955). The inhibitory activity of follicular fluid (Centola et al., 1981) appeared to come from the granulosa cells since these cells alone can inhibit oocyte maturation (Tsafriri and Channing, 1975; Sato et al.,
1982). The oocyte remains in the germinal vesicle stage under the influence of inhibitory factors produced by the granulosa cells (Stone et al., 1978; Tsonis et al., 1983; Lawrence et al., 1978). These factors are transferred in some manner interrupting the resumption of meiosis (Anderson and Albertini, 1976; Cameron et al., 1983). The inhibitory factor has been characterized as a peptide with a molecular weight of about 2,000 and its effect on oocyte maturation is not destroyed by heating to 60 C (Tsafriri et al., 1976). It has been shown that the inhibitory activity of the low molecular weight fraction was dependent upon intact cumulus cells since GVBD was suppressed in cumulus cell-enclosed, but not denuded oocytes (Hillensjo et al., 1979).

The oocyte is coupled to the surrounding follicle cells through highly developed system of gap-junctional contacts. The cells of the innermost layer of the cumulus (the corona radiata cells) extend numerous cytoplasmic processes through the intervening zona pellucida to contact the oocyte. Some of these processes are deeply inserted into the oocyte cortex (Anderson, 1974). These junctions mediate the entry of nucleotides, sugars, phospholipid precursors, and amino acid (Moor, 1983). The intercellular passage of these substances into the sheep oocyte remains at a high levels for the first 12 hours of maturation and then declines sharply in the following 3 hours. The tight relationship between the processes and the oocyte is lost during the final phase of
preovulatory maturation (Gillula et al., 1978) and the processes begin to retract or degenerate (Zambori, 1974). This relation is compensated for by a significant increasing uptake of substrates across the membrane itself (Moor and Smith, 1979). Degenerative changes in the junctions, induced by FSH rather than LH, account for the reduced transmission of substances into maturing oocytes (Moor and Cran, 1980). It has been suggested that the inhibitory factors located in the granulosa cells act through the cumulus-oocyte-complex to inhibit the production of mRNA necessary for GVBD (Sato et al., 1982). Dekel and Beers (1978) suggested that the disruption of junctional contact also prevents the entry of an inhibitor of meiosis into the oocyte and thereby initiates nuclear maturation. However, it is unlikely to apply to meiotic regulation in sheep oocytes since nuclear change precedes junctional disruption in this species (Moor and Heslop, 1981). Larsen et al (1986) reported that selective uncoupling of adjacent cumulus cells in rats occurs after injection of HCG but the heterologous coupling between the oocyte and the corona is still fully maintained. The corona-oocyte unit may therefore be isolated from the granulosa cells (source of inhibitory signals) at an early stage in maturation. Channing et al (1983) observed that the follicular fluid in large follicles has significantly less inhibitory activity than that in small follicles. In addition, small follicles contained
higher concentration of the low molecular FSH binding inhibitor (FSH-BI), suggesting that the low molecular FSH-BI plays a role in regulating follicular responsiveness in vivo (Sluss et al., 1983). A decline of the inhibitory activity in the follicle is a necessary prerequisite for the resumption of meiotic maturation in an adequately differentiated oocyte. The reduced sensitivity of oocytes may also reflect maturational changes occurring in the follicles after the gonadotropin surge (Tsafriri et al., 1977).

The resumption of the oocyte maturation is inhibited in the case of either the oocyte kept in contact with the granulosa cells or the oocyte surrounded by cumulus cells, suggesting that the inhibitory action on oocyte maturation appears to exist through the mediation of the cumulus cells. Sato et al. (1982) observed that oocytes were maintained in meiotic arrest when isolated cumulus-oocyte-complexes were cultured with follicular cells. The cumulus cells may have an important role in mediating the effect of the inhibitory activity of the granulosa cells (Cross and Brinster, 1970). The cumulus cells which surround the oocyte and the zona pellucida undergo morphological and functional changes during the process of in vivo and in vitro maturation. The development of morphological changes is referred to as cumulus maturation. Dissociation of the cumulus cells is a characteristic feature at this stage. Since the structural changes of the cumulus cells are correlated with oocyte
maturation (Zambori, 1974), the oocytes and cumulus maturation occur simultaneously during the process of meiosis (Dekel et al., 1979). The process of dissociation of the cumulus cells in the mouse is stimulated by FSH (Eppig, 1980). A high intrafollicular level of FSH stimulates production of estradiol which may be an important factor as well (Laufer et al., 1984). This process may also be involved in the deposition of a hyaluronic acid in the cumulus cells (Eppig, 1979). It has been hypothesized that cyclic adenosine monophosphate (cAMP) could be an intracellular inhibitor (Tsafriri and Channing, 1975) and involved in the regulation of oocyte maturation. Dekel and Beers (1978, 1980) observed that the oocyte in the ovary remained in the germinal vesicle stage by the passage of cAMP from cumulus cells to the oocyte through gap junctions. Since cAMP analogs blocked maturation of the oocyte in vitro, cAMP may play an important role in the arrest of meiotic maturation (Wassarman et al., 1976). Hamberger et al (1979) showed that granulosa cells isolated from follicles increase their production of cAMP in response to LH and FSH in vitro. An elevation of cAMP elevated in the isolated cumulus-oocyte-complex occurred when treated with FSH, but not with the denuded oocyte; as the result was a significant inhibition of GVBD (Schultz et al., 1983). Addition of cAMP analogs to the medium prevented both LH-induced maturation (Tsafriri at al., 1972) and the spontaneous maturation of the
oocyte (Magnusson and Hillensjo, 1977). It is possible that high intracellular levels of cAMP in the cumulus cells are responsible for the maintenance of meiotic arrest of the oocyte. Hamberger et al (1979) suggested that a drop of follicular cAMP levels before ovulation is an important step of the oocyte maturation process.

Exhaustive studies in the mouse indicate that a phosphoprotein, substrate of protein kinase, maintains meiotic arrest in mouse oocytes (Maller and Krebs, 1977; Bornslaeger et al., 1986). A decrease in intraoocyte cAMP and an accompanying decrease in protein kinase results in the consequent resumption of meiosis (Bornslaeger et al., 1986). In sheep oocytes, cAMP concentrations do not decline at the initiation of maturation but instead increase significantly at this time (Moor and Heslop, 1981). It appears that the increase in intraoocyte cAMP levels during maturation is initiated by gonadotropins acting on the follicular cells (Crosby et al., 1985). Signals from the follicular cells stimulate the adenylate cyclase system and consequently increase the cAMP concentration in the oocyte.

The addition of LH and FSH to the medium stimulated the production of cAMP (Kolena and Channing, 1972), however, cAMP appeared to stimulate maturation of the oocyte (Jagiello et al., 1975). The involvement of cAMP in the control of the resumption is that LH induces a rapid rise in follicular cAMP level (Marsh, 1976). It has been shown that
an increase in cAMP levels occurred in the follicular cells containing LH receptors (Bauminger et al., 1978). The granulosa cells from a large follicle produced a large amount of cAMP in comparison with cells from a small follicle when stimulated by LH (Schwartz and Channing, 1979). Since the increase in cAMP levels occurring in gonadotropin-stimulated granulosa cells is only transient (Lawrence, 1978), the progressive resumption of meiosis is induced soon after the gonadotropic surge (Salustri and Siracusor, 1983). The interpretation of the contradicting effects of cAMP on the oocytes has been explained by stating that a level of follicular cAMP is elevated temporarily by LH and the oocyte resumes meiosis, whereas the oocyte is inhibited from maturation when exposed to continuous high concentration of cAMP (Tsafriri, 1979). The other hypothesis suggested has been that the inhibitory factor generated or activated by cAMP is transferred through gap junctions from the cumulus cells to the oocyte (Eppig et al., 1983). Eppig and Downs (1984) reported that a relatively inactive form of inhibitory factor in the follicular fluid which transferred to the cumulus cells could be activated by cAMP. The inhibitory substance is small enough to traverse gap junctions and interact with a cAMP-dependent process in the cumulus cells. Since elevation of cAMP levels in the cumulus cells results in the inhibition of oocyte maturation, it is possible that the
resumption of the maturation is suppressed by the cAMP-dependent process although cAMP may not be transferred from the cumulus cells to the oocyte (Downs and Eppig, 1984). In any case, the metabolic changes of cAMP in the process of the inhibition and resumption of meiosis indicated a potentially paradoxical reaction (Dekel and Beer, 1980).

**Gonadotropic Influence on Meiotic Resumption**

The role of gonadotropins in the maturation of the oocyte has been widely acknowledged since evidence was presented and demonstrated resumption of meiosis when gonadotropins were added to the medium (Thibault et al., 1975; Tsafriri and Channing, 1975; Thibault, 1977). Experimental evidence for the concept of how gonadotropins influence the maturation of the oocyte has been reported. Since FSH and LH levels are elevated prior to ovulation, both hormones may be required to allow resumption of oocyte maturation (Dekel and Beer, 1978). In fact, maturation of the oocyte is induced by a specific stimulation of the preovulatory gonadotropic surge in vivo so that the combined effects of FSH and LH on their target cells and the metabolic interaction in the ovary must be considered (Eshkol et al., 1970). The relationship between the preovulatory surge of luteinizing hormone (LH) and the resumption of oocyte meiosis is well established (Edwards
et al., 1970; Seibel et al., 1982). Studies have shown that LH may neutralize the inhibitory action of follicular fluid and induce the resumption of meiosis (Tsafriri, 1976). The LH-induced meiotic resumption may involve an initial stimulation of the granulosa cells and exert an effect on the oocyte through the cumulus cells (Leibfried and First, 1980). It has been demonstrated that LH receptors were located in the granulosa cells and cumulus cells (Amsterdam and Tsafriri, 1979). They reported that receptor sites for LH were present in the granulosa cells and cumulus cells but the concentration of receptors was much higher in the granulosa cells. The addition of LH to the medium was beneficial to effect the onset of GVBD (Lopata et al., 1977) and to induce maturation of the oocyte (Tsafriri et al., 1973; Lindner et al., 1974). The inhibitory activity could be overcome by the addition of LH to the medium (Tsafriri et al., 1972) or contact of the follicle with LH (Ayalon et al., 1972). When gonadotropins, particularly FSH were added to the medium, cumulus-oocyte-complex facilitated the cumulus expansion (Ball et al., 1983) and a high proportion of oocytes were matured and fertilized (High et al., 1983). Laufer et al (1984) showed that the low level of gonadotropins was associated with failure of fertilization due to the incomplete nuclear and cytoplasmic maturation. The inhibition of oocyte maturation is mediated by the cumulus cells and the resumption of meiosis may be a result
of the stimulatory effect of gonadotropins on the cumulus cells (Dekel and Beer, 1978). Gonadotropins stimulated the cumulus cells to increase hyaluronic acid synthesis which interrupted the gap junction between the oocyte and cumulus cells (Sato et al., 1982). Immature cumulus-oocyte-complex is insensitive to hyaluronidase and the sensitivity of the cumulus cells increases in the ovary following the gonadotropic surge. Thus, gonadotropins induce separating of cumulus-oocyte-complex from the granulosa cells by releasing the cumulus-oocyte-complex from inhibitory substances in the granulosa cells. This event is characterized by modification or expansion of the cumulus cells and is associated with GVBD in the oocyte. The maturation of the oocyte surrounded by expanded cumulus cells was not inhibited although the oocyte was in contact with the granulosa cells; however, the oocyte surrounded by non-expanded cumulus cells was inhibited (Sato et al., 1982). Therefore, gonadotropins alter the metabolism of the cumulus cells and induce resumption of meiosis in the oocyte (Salustri and Siracuso, 1983) by interrupting this mode of inhibitory substances through the gap junction (Thibault, 1972; Ball et al., 1983). As follicles were incubated, FSH appeared to be an effective uncoupling agent for the cumulus cells (Moor, 1981) and stimulated the secretory activity of the granulosa cells (Tsafriri and Channing, 1975). FSH induces metabolic changes in follicular cells, resulting in
the mechanical separation between the oocyte and cumulus cells due to intercellular deposition of hyaluronic acid (Salustri and Siracuson, 1983). The mechanism for the control of oocyte maturation is the reduction of the intercellular coupling between the oocyte and cumulus cells (Dekel et al., 1981). However, it has been suggested that a change occurs in the signal communicated from the cumulus cells to the oocyte as a result of gonadotropic stimulation, but not by an interruption of the intercellular coupling (Gilula et al., 1978), since maturation of the oocyte is initiated before the termination of the coupling (Moor and Cran, 1980; Eppig, 1982). It has not been established whether gonadotropins directly induce oocyte maturation since the fact that the oocyte isolated from ovarian follicles undergoes meiotic maturation spontaneously in the medium containing with no gonadotropins (Eppig and Downs, 1984). A mechanical process may be responsible for the resumption of meiosis by isolating the oocyte from the granulosa cells (Foot and Thibault, 1969). The GVBD of isolated oocytes is achieved earlier than the corresponding change in hormone-induced maturation of oocytes. The contradiction of evidence may be interpreted as the oocyte being maintained by some regulatory factor which is essential for the meiotic process under physiological condition (Tsafriri, 1979).
Sperm Capacitation and Acrosome Reaction

Testicular spermatozoa of mammals develop fertilizing capacity as they pass through the epididymis. This process is referred to as the epididymal maturation of spermatozoa. Even after this maturation, spermatozoa require an additional phase of maturation within the female genital tract before they are able to fertilize oocyte (Yanagimachi, 1981). This is called capacitation. Epididymal maturation includes morphological, physiological and biochemical changes in the sperm components. The plasma membrane is the site of the most prominent changes during epididymal maturation. The sperm plasma membrane absorbs a variety of substances from its environment. Some of these come from the seminiferous tubule, epididymis, and vas deferens. The sperm surface is further coated with seminal plasma components until ejaculation. The removal or alteration of the coating materials is a part of capacitation. Some of these surface-coating materials are so tightly bound to the spermatozoa that they can not be readily eluded by simple washing with physiological saline (Scacciati and mancini, 1975). Oliphant and Brackett (1973) showed that antibodies raised against seminal plasma strongly agglutinate ejaculated rabbit spermatozoa. As the spermatozoa incubated in the uterus of the estrous female, they gradually become incapable of agglutination by the antibodies, with complete loss by 12 hours. This indicates that the seminal plasma
components on the sperm surface are gradually removed or altered as capacitation proceeds. Removal of the seminal plasma components from the sperm surface, however, can not be considered to represent the entire process of capacitation because both ejaculated and epididymal rabbit spermatozoa are functionally capacitated at the same rate (Overstreet and Bedford, 1974). The removal of materials that coat the sperm surface before the spermatozoa are exposed to seminal plasma is also important in terms of capacitation. Spermatozoa of some species (e.g. mouse, guinea pig and human) are known to gain capacitation when incubated in relatively simple media (Rogers, 1978). This indicates that sperm surface-coating substances in these species are readily removed by simply exposing them to media. Spermatozoa of some other species (e.g. pig, bull and monkey) are less readily modified their fertilizing capacity in artificial media. They require some more specific environment for modification of sperm surface. When rabbit (Brackett and Oliphant, 1975) and bull spermatozoa (Brackett et al, 1982) are exposed to hypertonic media (380 mOSM), an enhancement in the removal of the surface coat and facilitation of capacitation occurs. The genital tract of the estrous female provides the most favorable conditions for capacitation of spermatozoa of the same species. However, the condition of the female tract is not strictly species specific. The female tract of one species can capacitate spermatozoa of some other species.
For instance, the rabbit oviduct is capable of inducing capacitation of squirrel monkey and hamster spermatozoa (DeMayo et al., 1980).

The acrosome is a membrane-bound, caplick structure covering the anterior portion of the sperm nucleus. The acrosome is a structure analogous to a lysosome (Hartree, 1975) or a zymogen granule of pancreatic cells (Friend, 1977). It contains a variety of hydrolyzing enzymes such as hyaluronidase, proteinase, esterase, acid phosphatase, collagenase etc. (Stambaugh, 1978). In intact acrosomes, some of these enzymes such as acrosin, are not biologically active (Meizel and Muherji, 1975, 1976). Hyaluronidase and acrosin are the two acrosomal enzymes that have been most extensively studied (Morton, 1976, 1977; Stambaugh, 1978). Gould and Bernstein (1975) demonstrated that hyaluronidase is located predominantly within the matrix of the acrosomal cap. However, Morton (1975) believed that at least some residual hyaluronidase is bound to the inner acrosomal membrane. Green and Hochaday (1978), who labeled spermatozoa with antiacrosin antibodies, reported that acrosin is localized on the inner acrosomal membrane. This seems to support the theory that the bulk of acrosin activity is located on the inner acrosomal membrane (Anand et al., 1977). The acrosome, a relatively unstable structure, may be spontaneously disrupted during the senescence of spermatozoa as a result of harsh treatment. Such phenomena are called a
"false" acrosome reaction. It is clearly different from "true" acrosome reaction that occurs in actively motile spermatozoa (Meizel, 1978). The importance of the acrosome reaction in mammalian fertilization was recognized by Austin and Bishops (1958). They reported that the acrosomal caps of highly motile spermatozoa of the guinea pig and hamster were modified and then lost before spermatozoa begin to penetrates the zona pellucida. The acrosome reaction in mammalian spermatozoa involves membrane vesiculation or multiple fusion between the plasma and the acrosomal membranes (Piko and Tyler, 1964). The membrane vesiculation is usually confined to the acrosomal cap region (Bedford and Cooper, 1978) but the equational segment can also participate in vesiculation (McMaster et al., 1978).

Whether the membrane vesiculation in the acrosomal cap region occurs simultaneously over its entire area or starts from a certain point is not known. The speed of the acrosome reaction probably varies according to species and the condition of the medium surrounding the spermatozoa. Parrish et al. (1985) estimated that bovine spermatozoa require about 4 to 6 hours to penetrate bovine oocytes in vitro. The biological function of the acrosome reaction is the release or exposure of acrosomal enzymes to assist the passage of spermatozoa through the zona pellucida. The acrosome reaction also triggers a physiological change in the plasma membrane which renders the sperm plasma membrane
capable of fusing with the ovum plasma membrane (Yanagimachi, 1977;1978). In most in vitro experiments, a large number of spermatozoa are deposited around the ovum and the cumulus oophorus partially or completely disperse (due to hyaluronidase released from spermatozoa; Thompson et al., 1974). Spermatozoa with intact acrosomes may reach the surface of the zona shortly after insemination and spermatozoa undergo acrosome reactions on the zona. Acrosome-intact spermatozoa may bind to the zona of the cumulus freed ovum immediately after insemination and begin their acrosome reaction on the zona surface (Saling et al., 1979). Spermatozoa can undergo both capacitation and acrosome reaction directly on the zona surface before penetrating it. Under in vivo condition, spermatozoa probably complete their capacitation before contact with the cumulus cells and begin acrosome reaction shortly before their contact with the cumulus.

The surface of spermatozoa is coated with macromolecular substances. These substances seem to protect or stabilize the sperm plasma membrane (Bedford, 1970). During capacitation some or all of these surface molecules must be removed or modified, rendering the sperm plasma membrane capable of fusing with the underlying outer acrosomal membrane. A seminal plasma component (glycoprotein) added to the medium prevents rabbit spermatozoa from initiating acrosome reaction (Eng and Oliphant, 1978). How the seminal
plasma and other extrinsic macromolecular components inhibit the acrosome reaction is unknown.

**Extracellular Components for Acrosome Reaction**

$Ca^{++}$ in the medium has proven to be essential for the acrosome reaction (Singh et al., 1978, 1980; Dudenhausen and Talbot, 1982). Extracellular $Ca^{++}$ is necessary for initiation of the acrosome reaction, but not for progression and completion of capacitation (Yanagimachi, 1972). Other conditions or components of the medium that affect or may affect the acrosome reaction include $K^+$ (Misny and Meizel, 1980), $Mg^{++}$ (Rogers and Yanagimachi, 1976), energy sources such as pyruvate and glucose (Fraser and Quinn, 1981) and macromolecular like albumin (Davis, 1978). The addition of other agents including glycosaminoglycans (Lenz et al., 1983; Parrish et al., 1985), phospholipases (Llanos et al., 1982) and zona pellucida glycoproteins (Bleil and Wassarman, 1983) have been shown to promote an acrosome reaction. Yanagimachi (1969a) reported that bovine follicular fluid (BFF) contained two factors (dialysable and heat stable, non dialysable and heat labile) responsible for the induction of the capacitation and acrosome reaction of spermatozoa. The mouse spermatozoa capacitated in the medium containing BFF (Iwamatsu and Chang, 1969). Fukui et al. (1983) reported that frozen-thawed bull semen incubated with BFF resulted in a significantly high fertilization rate of bovine follicular
oocytes in vitro. Since a large volume of follicular fluid is released from the follicle at the time of ovulation, a part of this fluid may enter the oviduct and change the oviductal environment (Yanagimachi, 1969b). Many factors in follicular fluid have been believed to be necessary for inducing capacitation and acrosome reaction of spermatozoa in the hamster (Givatkin and Anderson, 1969) and the cow (Lorton and First, 1979) and human (Edwards et al., 1969). It has been reported that glycosaminoglycans in the female reproductive tract may be responsible for acrosome reaction (Lenz et al., 1983) and proteoglycan in BFF enhances the incidence of acrosome reaction (Fukui et al., 1983). Since the sperm-capacitating potency of the follicular fluid is induced when it was diluted with the medium, a high concentration of follicular fluid is necessary for an efficient capacitation and acrosome reaction (Yanagimachi, 1969b).

Interaction of Spermatozoa with the Cumulus Cell

Bronson and Hamada (1977) presented experimental evidence that the mouse cumulus cells, but not the ovum, secrete a substance that alters the characteristics of the sperm surface. Chemotactic attraction of spermatozoa by the cumulus appears to be of secondary importance for the sperm-cumulus encounter. The presence of an intact cumulus cells is obviously not an absolute necessity for
fertilization. As stated previously, the cumulus cells are likely candidates for the induction of the acrosome reaction of spermatozoa in vivo. In vivo, fertilizing spermatozoa very quickly pass through both the cumulus and the zona pellucida (Zamboni, 1972). The time of the fertilizing spermatozoa within the cumulus appears to be too short for initiation and completion of acrosome reaction. Because the matrix of the cumulus is composed predominantly of hyaluronic acid polymers (Piko, 1969) and the acrosomes of mammalian spermatozoa are generally rich in hyaluronidase, the involvement of acrosomal hyaluronidase in cumulus penetration by spermatozoa has been inferred for a long time. (Sustin, 1960, 1961). The acrosomal hyaluronidase is required for passage of spermatozoa through the cumulus. Antibodies raised against hyaluronidase effectively block both cumulus dispersion by spermatozoa and fertilization (Dunbar et al., 1976). The mechanism by which acrosomal enzymes are released from fertilizing spermatozoa is by fenestration of the sperm membranes during the acrosome reaction. Bedford (1972) found a few rabbit spermatozoa with apparently intact acrosomes in the close vicinity of the zona pellucida of the ovum that were still completely surrounded by cumulus cells, although most spermatozoa in the cumulus were undergoing or had completed the acrosome reaction. Not all spermatozoa within the cumulus have reacted acrosomes. Thus, acrosomal enzymes are possibly not essential for sperm passage through
the cumulus. It is very curious that bull spermatozoa which are very rich in hyaluronidase (Blandau, 1961), fertilize the cow ovum which quickly lose the cumulus cells after ovulation. The bovine ovum under normal in vivo condition appears to meet the spermatozoa immediately after it enters the oviduct. At this time, the ovum might still be surrounded by a compact cumulus or at least by the cumulus matrix. The freshly ovulated bovine ovum is surrounded by a transparent viscous material. If the material contains polymerized hyaluronic acid complexes, the acrosomal hyaluronidase in bull spermatozoa would have an important function in fertilization.

Sperm Penetration through the Zona Pellucida

A freshly ovulated ovum presents two or three barriers to spermatozoa: the granulosa cells, the cumulus oophorus, and the zona pellucida. The spermatozoa must penetrate these layers and arrive at the vitellin membrane for fusion with this membrane. It has been speculated that the plasma membrane or the inner acrosomal membrane recognizes the zona by terminal oligosaccharide residues, similar to an antigen-antibody interaction. Capacitated spermatozoa bind to both the inside and outside of isolated hamster zonas, suggesting that the binding ligands are located the entire zona surface (Rogers and Bentwood, 1982). These ligands are apparently altered after sperm penetration of the zona and
prohibits further binding of spermatozoa. Wasserman (1987) has identified and isolated three unique mouse zona glycoproteins. This is receptor, called ZP-3 (molecular weight 83,000) consists of a 44,000-dalton polypeptide chain, to which several asparagine-linked (N-linked) and serine-threonine-linked (O-linked) oligosaccharides are covalently attached. Each zona pellucida contains approximately a billion copies of ZP3 that are synthesized and secreted, along with two other zona pellucida glycoproteins (ZP1 and ZP2). At the time of fertilization, the spermatozoa binds to the outer margin of the zona pellucida. Sperm recognizes some molecular feature of ZP3 in a species-specific manner. After a period of binding at the zona surface, the spermatozoa progress through the zona matrix. In most species, the zona pellucida changes its biochemical properties as soon as the first spermatozoa penetrates this layer. This is called the "zona reaction" and the altered composition of the zona prevents passage of further spermatozoa. There is a critical block to polyspermic fertilization, that is, fertilization by more than one spermatozoa. This block to polyspermy is important because polyspermy leads to mostly triploid embryos (Jacobs et al., 1978).

The existence of a chemical or enzymatic mechanism by which spermatozoa soften or dissolve the zona material is expected. This hypothetical zonalytic agent is generally
referred to as the zonalysin (Austin, 1961). As the zona can be dissolved by acrosin (Meizel and Mukerji, 1976), acrosin has been suggested to be the zona lysin. The site of zona lysin is believed to be the surface of the inner acrosomal membrane (Brown et al., 1975). It exposes directly to the zona material as the spermatozoa passes through the zona. Bedford and Cross (1978) have questioned this because rabbit zona treated with wheat germ agglutinin become very resistant to trypsin and acrosin and yet remain readily penetrable by spermatozoa. A proteinase other than acrosin may be involved in zona penetration by spermatozoa (Srivasta et al., 1979). The acrosomal hyaluronidase may also serve as a zona lysin (Perreault et al., 1980). Morton (1976) reported that some of the hyaluronidase is bound to the inner acrosomal membrane and functions as a zonalysin.

The mechanisms by which the acrosome reaction renders the spermatozoa capable of fusing with the ovum plasma membrane of either the equational segment or the postacrosomal region (or both) of the spermatozoa changes radically as a result of the acrosome reaction. The inner acrosomal membrane does not fuse with the ovum membrane but this area engulfed by the ovum microvilli. The remaining components of the sperm tail are incorporated into the ooplasm and the midpiece, mitochondria, and axial filament are disassembled. The surface of the mammalian ovum has numerous microvilli except for the area over the first polar.
body. Sperm ovum fusion seldom ever occurs in this area (Johnson et al., 1975).

**Cortical Granule Breakdown and Block to Polyspermy**

Sperm-ovum fusion initiates structural and functional changes in the ovum in order to help prevent polyspermy. The most dramatic structural change is the exocytosis of the cortical granules (Soupart and Strong, 1975). The material contained within the cortical granules is released into the perivitelline space. The block to polyspermy involves changes in both the vitelline membrane and the zona pellucida (Menezes and Peter, 1985). It appears that the initiating mechanism for cortical granule release is similar to that of other secretory cells. Under natural conditions, the fusion of spermatozoa with the vitelline membrane probably elicits membrane changes. Granule discharge starts at the point of sperm entry and appears to propagate around the entire ovum (Wassarman, 1987). This phenomenon that changes the magnitude of the membrane potential is called membrane-depolarization (McCulloh et al., 1983). Sperm fusion causes a change in calcium permeability and/or localization that initiates the granule-secretion and activate various metabolic pathways in the ovum cytoplasm. The cortical granule reaction releases granule constituents into the perivitelline space and the cortical granule membranes fuse with the vitelline membrane so that a new
membrane covers the ovum surface (Rogers and Bentwood, 1982). These actions are thought to be the sources of the polyspermic block. Cortical granule contents are thought to induce the zona reaction directly and destroy sperm-binding affinity. The active components may be trypsin-like proteases and glycoprotein substances that are present in abundance (Gwatkin et al., 1973). The neuraminidase-like enzyme of the sperm acrosome also cause hardening of the zona, that is, a decrease in digestibility by enzymes.

When immature ovarian oocytes at the germinal vesicle stage (for the mouse, rat, hamster, and rabbit) are inseminated in vitro, spermatozoa are commonly found in the perivitelline space. However, swollen (decondensing) sperm heads are seldom seen within the cytoplasm (Niwa and Chang, 1975). This had led to conclusion that immature oocytes are not penetrated or are barely penetrated by spermatozoa. The membrane fusion between ovum and spermatozoa occurs normally, but decondensation of sperm nuclei fails to occur (Berrios and Bedford, 1979). Thus, the plasma membrane of the ovum of the hamster and perhaps all other mammalian species seems to have capacity to fuse with the sperm membrane even before the oocyte completes nuclear maturation.

**Pronucleus Formation**

As the spermatozoa contacts and penetrates the vitelline membrane, the ovum completes its second meiotic division and
extrudes the second polar body. The final stage of gamete interaction is the formation of a male pronucleus (from the sperm chromosomes) and a female pronucleus (from the ovum chromosome). Hyttel et al (1987) described that the female pronucleus formation was characterized by condensed chromatin configuration in the cytoplasm. The subsequent stage is that of an increasing investment of the female chromatin by a nuclear envelope developed from the smooth endoplasmic reticulum. The final stage was characterized by complete investment of the chromatin by the nuclear envelope. A similar sequence was observed during the male pronucleus formation, which was initiated by the sperm head stage characterized by a naked decondensing sperm head in the cytoplasm. The nuclear envelope of the sperm nucleus disappears and decondensation of the nucleus begins. A new nuclear envelope then appears around the pronucleus (Bedford, 1972). DNA synthesis in both sperm and ovum nuclei is more or less synchronous with the development of pronuclei (Krishna and Generous, 1977). Male and female pronuclei come into close proximity in the center of the ovum and the chromosomes of both pronuclei mingle for the first mitotic division (Anderson et al., 1975). The Male nucleus incorporated into the cytoplasm of mature ovum soon begins to decondense. It is possible to predict that chemicals or factors in the ovum cytoplasm induce nuclear decondensation. Oocytes from ovaries of the rabbit and cow
matured in vitro were able to incorporate spermatozoa. Male nucleus, however, failed to decondense to the full extent. The oocytes matured in vitro lacked some factors necessary for decondensation of the sperm nucleus. This factor has been referred to as the male pronucleus growth factor (MPGF). There appears to be an interplay of this factor within the ooplasm that controls pronuclear formation.

The occurrence of abnormalities in oocytes during fertilization is the most obvious result of incomplete maturation (Thibault, 1977). The cytoplasmic factors required to decondense sperm chromatin is absent in sheep oocytes fertilized during the germinal vesicle or metaphase I stage of meiosis. Quantitative analysis of polypeptide patterns indicate that the ability to decondense sperm chromatin is associated with the synthesis of a small group of polypeptides between 12 and 18 hours after the indication of maturation (Osborn and Moor, 1983). Yanagimachi and Usui (1972) reported that hamster sperm nucleus incorporated into the cytoplasm of immature ovum but the male pronucleus were unable to decondense in the ovum. The male nucleus incorporated into the cytoplasm of the mature ovum in vivo, on the other hand, rapidly decondensed. It was also predicted that the cytoplasm of the immature hamster ovum lacks some factors necessary for decondensation of the sperm nucleus. They called this factor, sperm nucleus decondensing factor (SNDF). Usui and Yanagimachi (1976) inferred that
SNDF begins to appear in the ovum cytoplasm after breakdown of the germinal vesicle, increases in amount as oocyte maturation advances, and reaches a maximum level shortly before or after ovulation. The origin of SNDF is unknown. Usui and Yanagimachi (1976) speculated that SNDF is synthesized in the oocyte cytoplasm accumulated in the germinal vesicle, and then released onto the oocyte cytoplasm at the time of germinal vesicle breakdown. The mechanisms of pronucleus development are not fully known. When a large number of spermatozoa enters an ovum, the sperm nucleus decondense but fail to develop into pronucleus. Formation of the female pronucleus occurs by a similar process as a result of activation but does not require the decondensation step (Rogers and Bentwood, 1982). However, the ovum chromosomes develop into a pronucleus with distinct nucleoli (Hirao and Yanagimachi, 1979). They suggested that the cytoplasmic factor for development of the female pronucleus is not the same as that which controls the development of the male pronucleus. Following the appearance of the nucleoli, there is a period of DNA synthesis (chromosome replication). Fusion between the pronuclei is facilitated by an interdigitation of numerous projection from the nuclear envelopes. The chromatin of both nuclei then condense, and the chromosomes organize at a metaphase plate for the first zygotic division, thus completing the fertilization process.
CHAPTER III

MATERIALS AND METHODS

Experimental Design

I. Maturation of bovine follicular oocytes

The effects of serum supplements and gonadotropins on cumulus cell expansion and stage of nuclear maturation of bovine follicular oocytes were determined after incubation for 26 to 28 hours.

(1) Serum supplements

A. Ham's F-10 + 5 mg/ml BSA
B. " + 10% follicular fluid (FF)
C. " + 10% estrous cow serum (ECS)
D. " + 10% fetal calf serum (FCS)
E. Ham's F-10 only
F. Control (not cultured)

(2) Serum and FSH supplement

A. Ham's F-10 + 5 mg/ml BSA + 1 ug/ml FSH
B. " + 10% FF + 1 ug/ml FSH
C. " + 10% ECS + 1 ug/ml FSH
D. " + 10% FCS + 1 ug/ml FSH

(3) Serum and HCG supplement

A. Ham's F-10 + 5 mg/ml BSA + 2 IU HCG
B. " + 10% FF + 2 IU/ml HCG
C. " + 10% ECS + 2 IU/ml HCG
D. " + 10% FCS + 2 IU/ml HCG

(4) Serum, FSH and HCG supplement
A. Ham's F-10 + 5 mg/ml BSA + 1 ug/ml FSH + 2 IU/ml HCG
B. " + 10% FF + 1 ug/ml FSH + 2 IU/ml HCG
C. " + 10% ECS + 1 ug/ml FSH + 2 IU/ml HCG
D. " + 10% FCS + 1 ug/ml FSH + 2 IU/ml HCG

II. Fertilization of bovine follicular oocytes
The effects of ECS or FCS and gonadotropin supplementation on bovine follicular oocytes fertilized in vitro were determined (gonadotropins were only used during oocyte maturation, not during fertilization).

(1) Serum supplement
A. Ham's F-10 + 10% ECS
B. " + 10% FCS

(2) Serum and FSH supplement
A. Ham's F-10 + 10% ECS + 1 ug/ml FSH
B. " + 10% FCS + 1 ug/ml FSH
(3) Serum and HCG supplement
A. Ham's F-10 + 10% ECS + 2 IU/ml HCG
B. " + 10% FCS + 2 IU/ml HCG

(4) Serum and FSH, HCG supplement
A. Ham's F-10 + 10% ECS + 1 ug/ml FSH + 2 IU/ml HCG
B. " + 10% FCS + 1 ug/ml FSH + 2 IU/ml HCG

Preparation of glassware

All glassware was rinsed with tap water and placed into a solution of 1% 7X Tissue Culture Soap. The soap solution was heated to between 80 and 85 C. This enhances the cleaning ability of the soap and remove any adhering debris. The soap solution was allowed to cool to between 40 and 45°C. The glassware was scrubbed and rinsed with tap water and then allowed to air dry for 10 to 15 minutes. The glassware was placed into a 10% stock solution of 7X Tissue Culture Soap overnight. After soaking, the glassware was rinsed with tap water and placed into double distilled water for 1 to 2 hours. The glassware was rinsed 6 times with double distilled water and allowed to air dry for 10 to 15 minutes. Finally, the glassware was wrapped with plastic wrap and autoclaved for 30 minutes at 121 C (at 17-18 psi).

1) Flow Laboratory Inc., Mclean, Virginia 22102
Oocyte Collection

Ovaries were obtained from a local slaughter house and transported to the Theriogenology Research laboratory. Ovaries were collected from cows within 10 minutes after death of donor and placed in 0.9% saline at 37°C for not more than 2 hours by which time oocytes were harvested. This period may play a critical role in determining if an oocyte is capable of subsequent meiosis in vitro. Shea et al. (1982) reported that a 2-hour elapse from death to oocyte recovery from the follicle resulted in no apparent affect on maturation of bovine oocyte. Oocytes were aspirated only from 3 to 8 mm diameter follicles through the ovarian stroma using a sterile 6 ml syringe with a 20 gauge needle attached. Repeated aspiration of follicles were performed to collect oocytes into the syringe. These oocytes were placed immediately into a sterile 35 mm diameter petri dish and washed 3 times with medium. The optimum pH in the medium for bovine oocyte is believed to be 7.2 to 7.4 and the exposure of medium to air may increase the pH. Oocytes were transferred to pre-equilibrated fresh medium immediately during each step of washing other than when they were in the medium under an atmosphere containing 

2) The Herman Falter Packing Co., Columbus Ohio
3) Corning Glass Works, Corning, N.Y. 14831
5% CO2. Normal immature oocytes were defined as those with a thick layer of cumulus cells which is firmly attached to the oocyte. All oocytes obtained were cultured unless morphological degenerations were observed.

Preparation of Medium

Modified Ham’s F-10 medium consisted of Ham’s F-10\textsuperscript{4} to which had been added 0.48 mg/ml NaHCO3, 0.50 mg/ml KHCO3, 0.25 mg/ml Calcium Lactate, 100 IU/ml Penicillin, and 100 IU/ml Streptomycin.

Preparation of serum and gonadotropin supplements

Blood (100 ml) was taken from the jugular vein of a cow at the time of standing estrus. The blood was collected into 10 ml tubes with no anticoagulant added, allowed to clot at 30°C for 2 hours, and centrifuged. The serum was aspirated and placed in 10 ml vials and stored at -20°C. Prior to its addition to the medium, the serum was heated to 56°C in a water bath for 30 minutes. The heat treated serum was passed through a filter (0.22 um)\textsuperscript{5} for sterilization.

4) GIBCO Laboratory, Gland Island, NY. 14072
5) Millipore Co., Bedford, MA. 01730
Fetal calf serum and crystallized bovine serum albumin\textsuperscript{6} were used as the medium supplements. The bovine follicular fluid was collected from preovulatory follicles (more than 15 mm in diameter) and poured into 10 ml vials. The follicular fluid was centrifuged for 30 minutes at 1000 g to remove cellular debris. The supernatant fluid was filtered (0.22 um) and placed into 3 ml vials. The follicular fluid was stored at -20°C in a freezer. The follicular fluid was heated to 56°C in a water bath for 30 minutes before use. Follicle stimulating hormone\textsuperscript{7} and chorionic gonadotropin\textsuperscript{8} were used as gonadotropic supplement.

**Culture of oocyte for maturation**

Oocytes were cultured in the modified Ham’s F-10 medium supplemented with 5 mg/ml BSA (A), 10% follicular fluid (B), 10% estrous cow serum (C), 10% fetal calf serum (D) and 1 ug/ml FSH or 2 IU/ml HCG at 38.5°C in an atmosphere of 5% CO\textsubscript{2}, 5% O\textsubscript{2}, balanced N\textsubscript{2} and 100% relative humidity for 26 to 28 hours.

\textsuperscript{6} Sigma Chemical Co., St. Louis, MO. 63178

\textsuperscript{7} Schering Co., Kenilworth, NJ. 07033

\textsuperscript{8} LyphoMed Inc., Melrose Park, IL. 60160
Staining of oocytes

After culturing, all oocytes were fixed and stained. Cumulus cells were removed with hyaluronidase (300 units/ml in PBS) and mechanical disturbance by suction and release of the oocyte in small bore pipettes. The oocyte in a drop of medium was pipetted onto a micro slide. A coverslip with inert stopcock grease spots at each of its four corners was placed directly over the center of the drop of solution. While observing under 100X magnification, the coverslip was pressed down on the oocyte until it was held firmly in place. The fixation of oocytes was carried out by placing the slides in a fresh mixture of acetic acid and methanol (1:3) overnight. The preparation was stained with a drop of acetic-orcein (2% orcein in 60% acetic acid) for a few minutes and washed under tap water. The edges of the coverslip were sealed with fingernail polish to prevent the preparation from drying. Both low power and oil immersion were used for detailed examination.

Criteria of maturation

The oocytes were classified based on their meiotic progress as follows based on the criteria described by Shea et al. (1976).

I. Germinal vesicle stage

An intact nuclear membrane with the chromatin of a meiotically inactive primary oocyte.
II. Metaphase I
The nuclear membrane broken and a chromatin pattern characteristic of an oocyte resuming meiosis present.

III. Metaphase II
A polar body present within the perivitelline space and the maternal chromatin complement identified in the oocyte.

IV. Degenerate
An oocyte with obvious degenerative changes such as a cytoplasm which was vacuolated, shrunken or fragmented or a chromatin complement which was scattered or highly condensed.

Preparation of Sperm
A highly fertile semen sample (0.25 ml of cryopreserved semen)\(^9\) was selected based upon conception rates exceeding 60\%. One straw was thawed and diluted to 2.0 ml with the modified Ham’s F-10 medium in a 10.25 x 64 mm culture tube and incubated for 5 minutes. The tube was then centrifuged at 300 g twice for 5 minutes each. The supernatant solution was discarded and the sperm were resuspended in 1.0 ml medium containing 25\% heat-treated follicular fluid and incubated at 38.5\°C in an atmosphere of 5\% CO\(_2\), 5\% O\(_2\),

\(^9\) Select Sires, Plain City, Ohio
balanced N2 and 100% relative humidity for four hours. The top 0.85 ml of medium from the tube was removed and transferred to a 10.25 x 64 mm culture tube. The number of sperm was counted with a hemocytometer and recorded. Separated sperm were diluted to 25 x 10^6 sperm/ml with the same medium.

**Co-culture of oocytes and sperm**

An aliquot of sperm suspension (2 ul) was added to 50 ul of medium containing 5 to 7 oocytes. The medium was covered with sterile paraffin oil (S.V. 125/135). Oocytes and sperm were co-cultured at 38.5°C in an atmosphere of 5% CO2, 5% O2, balanced N2 and 100% relative humidity for 24 hours. At the end of this culture period, oocytes were stained (the same as described above) and examined under a microscope for analysis of fertilization.

**Criteria of fertilization**

Oocytes were classified based on the criteria described by Leibfried-Rutledge et al. (1987).

I. Sperm in the oocyte cytoplasm without pronucleus development

II. A single pronucleus in the oocyte cytoplasm.

III. Two pronuclei with or without sperm tail remnants

IV. Polyspermy: more than one sperm heads or more than two pronuclei in the oocyte cytoplasm.

V. Degenerate: fragmented or scattered chromatin complement in the oocyte cytoplasm.

Gel electrophoresis

Each serum supplement was evaluated by polyacrylamide gel electrophoresis (PAGE) in the method described by Laemmli (1970). Aliquot of BSA, FF, ECS, and FCS in a sampling buffer was boiled for 3 minutes and electrophoresed on 15.0% acrylamide gels. The sampling buffer consisted of 20 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 40% Glycerol, 0.02% Bronophenol blue, 2% Mercaptoethanol. The acrylamide gel consisted of 4.5 ml lower stock (1.5M Tris-HCl, pH 8.8, 0.4% SDS), 9.0 ml acrylamide stock (30% acrylamide, 0.8% Bis), 0.2 ml glycerol, 4.2 ml distilled water, 30 ul 10% ammonium persulfate, and 7 ul TEMED. Gels were run at a constant current of 4 mm amp per gel by the bronophenol blue marker migrating to within a few mm of the terminal gel end. Following electrophoresis, gels were both fixed and stained overnight with 0.25% coomassie brilliant blue R-250 in 10%

11) Bio-Rad Laboratory Richmond, CA. 94804
acetic acid (v/v) and 25% methanol (v/v) in water. Gels were destained in 10% acetic acid (v/v) and 10% methanol (v/v) in water. Samples were scanned in an Ultrascan XL, Laser Densitometer\textsuperscript{12} and mobilities of individual bands were expressed from densitometer tracings. A total protein concentration of each supplement was determined by the biuret method. Each 0.1 ml sample was diluted in 0.9 ml distilled water and added 4.0 ml copper sulfate solution (1.5 mg/ml copper sulfate, 30 mg/ml NaOH). The samples were retained for 10 minutes at room temperature for performing the reaction. The concentration of protein in each sample was measured by Spectrophotometer. The relative percent of BSA in FF, ECS, and FCS sample was also determined by a scanning and computing densitometer. The BSA concentration in each sample was computed from the standard curve determined by using the different concentrations of BSA. The concentration of albumin in the medium of each group was calculated by multiplying the total protein concentration by the relative percent.

**Electron Microscopic examination**

Oocytes were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer overnight at 4°C. The samples were given

\textsuperscript{12} LKB Bromma, Sweden
revised 3 times for 15 minutes each in 0.1 M Phosphate buffer. The oocytes were then stained in 1% osmium tetroxide (OsO4) in 0.1 M PBS for 2 to 3 hours at 4°C. The samples were again 3 rinses of 15 minutes duration each in 0.1 M PBS. Specimens were started into a dehydration series at room temperature. Dehydration was followed by 4 changes of 15 minutes duration each of ethanol (50, 75, 95, and 100%). Oocytes were then transferred into 100% acetone for further dehydration. Oocytes were embedded in Epon 812 and placed in a 65°C oven for 2 to 3 days. The samples were sectioned on a 2088 Ultratome V and recovered onto 500 mesh copper grids. The grids were dried overnight. The samples were stained in a uranyl acetate solution for 1 hour and rinsed in distilled water for 3 times and stained in a 0.34% lead acetate solution for 20 minutes. The samples were examined on a H300 transmission electron microscope.

Examination of acrosome reaction

The acrosome was examined by the method described by Watson (1975). A drop of semen was taken in every hour up to 4 hours and smeared on a pre-warmed slide and dried in

13) LKB Instruments Gaithersburg, Maryland
air. The smears were fixed by immersion in buffered formal saline\(^\text{14}\) for 20 minutes and washed in running tap water for 15 minutes. The smears were dried and then immersed in the modified Giemsa staining solution\(^\text{15}\) for 4 hours. They were rinsed in distilled water and dried. Stained spermatozoa were examined with a microscope equipped with an oil-immersion lens. The percentage of acrosome reacted spermatozoa was calculated for 100 spermatozoa selected at random on each slide. The progression of the acrosome reaction was classified by estimating the degree of change in acrosome into the following 4 groups; (1) Intact acrosome, the stain was clearly and evenly distributed over the spermatozoa anterior portion. (2) Multiple fusions between the plasma and outer acrosomal membranes; vacuolated membranes are visible. (3) The reaction was progressed; more membrane fusions are observed. (4) The reaction is complete; acrosome entirely lost.

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14) The Ohio State University Reagent Laboratory, Columbus, OH

15) Sigma Chemical Co. St. Louis, MO.
**Statistical analysis**

For statistical evaluation of the data, Chi square analysis was utilized. The data were organized into 2 X n contingency tables. Chi square values were calculated. When the resulting Chi square values were significant (P<0.05, 0.01), the Bouferroni procedure as a post test was used to determine whether the values were statistically different.
CHAPTER IV

RESULTS

The influence of bovine serum albumin (BSA), bovine follicular fluid (FF), estrous cow serum (ECS), or fetal calf serum (FCS) and their interaction with gonadotropins on the in vitro maturation and fertilization of bovine oocytes was determined.

Cumulus cell expansion of oocytes

After collection, oocytes were examined according to their morphological features. Normal oocytes appeared with a thick layer of tightly-bound cumulus cells (Figure 15) whereas degenerating oocytes had clumping granules and scattered cumulus cells (Figure 16). The oocytes were cultured in the medium with different serum and gonadotropin supplement for 26 to 28 hours. At the end of maturation culture period, all oocytes were examined and classified based on the structure of cumulus cell expansion, non-expansion, and degeneration. The most significant change of the oocyte was characterized by expansion of cumulus cells (Figure 17). Degenerating oocytes had a scattered chromatin complement and condensed, non-expanded
cumulus cells (Figure 18).

Of 73 (Ham's F-10 only), 159 (BSA), 93 (FF), 165 (ECS), 123 (FCS) oocytes, 10 (13.7%), 82 (51.6%), 49 (52.7%), 106 (64.2%), 63 (51.2%) of oocytes respectively were observed in cumulus cell expansion (Table 1). As shown in Figure 1, Ham's F-10 medium in the absence of serum components supported cumulus expansion less than that in the presence of serum components. All serum supplements increased the proportion of oocytes undergoing cumulus expansion (P<0.05). Cumulus expansion was higher in group C (ECS) compared with group A (BSA; P<0.05).

When FSH was added to the medium, of 135 (BSA), 108 (FF), 120 (ECS), 146 (FCS) oocytes, 77 (57.0%), 67 (62.0%), 86 (71.7%), 106 (72.6%) of the oocytes, respectively, were observed in cumulus expansion (Table 2). Significant differences (P<0.05) were found between group A (BSA) and group C (ECS), D (FCS; Figure 2). The increase (P<0.01) in percent cumulus expansion of oocytes cultured with FSH from the control level (serum supplement only) was observed only in group D (FCS; Figure 1 vs. Figure 2).

When HCG was added to the medium, of 64 (BSA), 99 (FF), 88 (ECS), 81 (FCS) oocytes, 36 (56.3%), 51 (56.0%), 59 (67.0%), 58 (71.6%) of the oocytes, respectively, were observed in cumulus expansion (Table 3). There was no significant difference between group A (BSA) and the other 3 groups (Figure 3). Group D (FCS) with HCG improved in
cumulus expansion significantly (P<0.01) compared with no gonadotropins.

When FSH plus HCG was added to the medium, of 109 (BSA), 126 (FF), 146 (ECS), 146 (FCS) oocytes, 60 (55.1%), 92 (73.0%), 101 (69.2%), 114 (78.1%) of oocytes, respectively, were observed in cumulus expansion (Table 4). There was a significant difference between group A (BSA) and the other 3 groups (Figure 4). Group B (FF) and D (FCS) with FSH plus HCG improved significantly compared with their groups with no gonadotropins. The percent of cumulus expansion was not improved in group A (BSA) and C (ECS) when gonadotropins were added to the medium.

The result of the effect of serum and gonadotropins supplement on the degenerative frequency of cumulus cells is given in Figure 5. When serum was added to the medium, degenerative frequency of cumulus cells during maturation significantly decreased in all groups except A (BSA). The viability of cumulus cells improved significantly by the addition of gonadotropins to the medium in group A (BSA) and C (ECS).

Germinal vesicle breakdown of oocytes

After examining the morphological features of cumulus cells, all oocytes were fixed and stained for analysis of the meiotic developmental stage. A normal oocyte consists of a spherical, evenly distributed zona pellucida and a
rounded vitelline membrane in close proximity to the zona pellucida. The oocyte was stained evenly and the maternal chromatin complement was clearly recognized in the oocyte (Figure 19). Whereas, a degenerative oocyte was vaculated and chromatin complement was scattered in the cytoplasm (Figure 20). Oocytes were classified based on their meiotic progress: (1) Germinal vesicle stage (Figure 21, 22), (2) Metaphase I (Figure 23), (3) Metaphase II (Figure 26) and (4) Degenerate (Figure 20). Of 117 oocytes examined after collection as controls (no culture), 114 (97.4%) of oocytes were at the germinal vesicle stage. This indicated that most of oocytes from follicles 3 to 8 mm in diameter remained at this immature stage.

Of 60 (Ham's only), 156 (BSA), 93 (FF), 125 (ECS), 113 (FCS) oocytes, 18 (30.0%), 59 (37.8%), 49 (52.7%), 76 (60.8%), 79 (69.9%) of oocytes, respectively, were observed in germinal vesicle breakdown (GVBD-Metaphase I and II; Table 5). Group B (FF), C (ECS), and D (FCS) were significantly different from group A (BSA) and Ham's only but there was no difference between group A (BSA) and Ham's only (Figure 6). There was significant difference for metaphase II when compared between A (BSA) and other 4 groups. The necessity for the presence of serum supplement to support oocyte maturation was demonstrated by this result. BSA added to the medium; however, did not improve oocyte maturation effectively.
When FSH was added to the medium, of 151 (BSA), 101 (FF), 132 (ECS), 114 (FCS) oocytes, 98 (64.8%), 64 (63.3%), 87 (65.9%), 77 (67.6%) of oocytes, respectively, were observed in GVBD (Table 6). There was no significant difference among groups but significant for metaphase II when compared between group A (BSA) and D (FCS; Figure 7). Only group A (BSA) was significant for GVBD and metaphase II when FSH added to the medium (P<0.01).

When HCG was added to the medium, of 62 (BSA), 83 (FF), 86 (ECS), 77 (FCS) oocytes, 33 (53.3%), 43 (51.8%), 56 (65.2%), 51 (66.2%) of oocyte respectively were observed in GVBD (Table 7). There was no difference among groups (Figure 8). Only group A (BSA) was significantly improved for GVBD and metaphase II when HCG added to the medium (P<0.05).

When FSH plus HCG was added to the medium, of 102 (BSA), 124 (FF), 128 (ECS), 117 (FCS) oocytes, 64 (62.7%), 78 (63.0%), 89 (69.5%), 80 (68.4%) of oocytes were observed in GVBD respectively (Table 8). There was no significant difference for GVBD among groups but significant for metaphase II when compared between group A (BSA) and other 3 groups (Figure 9). Only group A (BSA) was significant for GVBD and metaphase II when FSH plus HCG was added to the medium (P<0.01).

The effect of serum and gonadotropins supplement on the degeneration frequency of oocytes is given in Figure 10. Serum supplement decreased degenerative frequency of
oocytes but was significant only in group D (FCS). Gonadotropins added to the medium improved the viability of oocytes significantly in group A (BSA), B (FF), and C (ECS).

**Microscopic observation of matured oocytes**

In vitro matured bovine oocytes with well expanded cumulus cells examined by electron microscopy showed some characteristic features of cytoplasmic maturation. In the oocyte with well expanded cumulus cells, the cumulus cell projection penetrated the zona pellucida and ended in invaginations of the cytoplasmic membrane (Figure 27). The enlarged perivitelline space and developed microvilli projections into it were observed (Figure 27,28). The cortical granules were closely associated with the cytoplasmic membrane (Figure 28). The mitochondria were located in clusters and close to the smooth endoplasmic reticulum (Figure 29).

**Fertilization of bovine oocytes in vitro**

The effects of ECS or FCS and gonadotropin supplement on fertilization of bovine oocytes in vitro were examined. Oocytes were cultured with gonadotropins for maturation using the same as procedure described for oocyte maturation. After the end of culture period for maturation, oocytes were transferred to fresh medium without gonadotropins. After the oocytes and spermatozoa had been incubated together for
24 hours, the oocytes were fixed and stained for analysis of fertilization. Oocytes were classified as follows: (1) Sperm in the oocyte cytoplasm without pronuclear development (Figure 29), (2) A single pronucleus in the oocyte cytoplasm (Figure 30), (3) Two pronuclei with or without sperm tail remnants (Figure 31-32), (4) Polyspermy: more than one sperm or more than two pronuclei in the oocyte cytoplasm (Figure 33-34), (5) Degenerate: Fragmented or scattered chromatin complement in the cytoplasm. The incidence of sperm penetration was increased by gonadotropins. As shown in Figure 11, gonadotropins significantly increased the frequency of sperm penetration in oocytes cultured with ECS. In group II (FCS), there was a significant difference in (3) HCG compared with no gonadotropins. Oocytes in medium supplemented with FCS and FSH or FSH plus HCG; however, did not exhibit an increased percentage of sperm penetration compared with those in medium supplemented with no gonadotropins. When comparisons were made between groups (ECS vs. FCS) for the effect of gonadotropin supplement, there was a significant difference in (4) FSH plus HCG. FSH and HCG added to the medium significantly improved the frequency of pronucleus formation in both groups when compared with no gonadotropins (Figure 12). A comparison of the effect of serum and gonadotropin supplement on the incidence of sperm penetration and pronucleus formation is presented in Table 9 and 10.
Acrosome reaction of bovine spermatozoa in vitro

Acrosome reaction of spermatozoa was examined during the preincubation period before attempted fertilization (Figure 13). Acrosome reacted sperm were classified into four groups: (1) Intact acrosome (Figure 35), (2) multiple fusion between the plasma membrane and outer acrosome membrane (Figure 36), (3) The reaction in progress; more membrane fusions (Figure 37), (4) complete acrosome reaction (Figure 38). Acrosome reaction of sperm considerably increased in the medium containing bovine follicular fluid and 82.4% of acrosome reacted sperm were observed after 4 hours in culture (Table 11).

Protein analysis

A total protein concentration of each serum supplement was measured by the biuret method. The results in Table 12 showed that the total protein concentration in the medium of group A (BSA), B (FF), C (ECS), D (FCS) was 5.0, 8.2, 9.4, 5.0 mg/ml, respectively. Protein concentration of the follicular fluid and estrous cow serum was relatively high compared with fetal calf serum. Albumin concentration was evaluated by polyacrylamide gel electrophoresis (Figure 14) and calculated from the albumin standard curve. A total concentration of albumin in the medium of group A (BSA), B (FF), C (ECS), D (FCS) was 5.0, 2.4, 3.4, 2.5 mg/ml,
respectively. The follicular fluid contained relatively low level of albumin (29.7%) compared with estrous cow serum (35.8%) and fetal calf serum (50.0%).
Figure 1

Influence of supplemental serum components on the in vitro cumulus expansion of bovine follicular oocytes. Significant difference when compared with Ham's F-10 only (*P<0.05, **P<0.01) and group A (BSA; +P<0.05)
Figure 2

Influence of supplemental serum components and FSH on the in vitro cumulus cell expansion of bovine follicular oocytes. Significant difference when compared with group A (BSA; P<0.05)
Figure 2

Cumulus Expansion

% 100

0 50

Serum Supplement

(A) BSA

(B) FF

(C) ECS

(D) FCS

*
Figure 3

Influence of supplemental serum components and HCG on the in vitro cumulus expansion of bovine follicular oocytes. No significant difference when compared with group A (BSA; P<0.05)
Figure 3
Figure 4

Influence of supplemental serum components and FSH plus HCG on the in vitro cumulus cell expansion of bovine follicular oocytes. Significant difference when compared with group (A) BSA (*P<0.05, **P<0.01)
Figure 4

Cumulus Expansion

% 50 100

Serum Supplement (A) BSA (B) FF (C) ECS (D) FCS

Figure 4
Supplemental serum components and gonadotropins influence on degenerative frequency of bovine cumulus cells during in vitro maturation. Significant difference when compared with no gonadotropins in each group (*P<0.05, **P<0.01)
Figure 5
Figure 6

Influence of supplemental serum components on the in vitro germinal vesicle breakdown (GVBD) of bovine follicular oocytes. Significant difference when compared with Ham’s F-10 only (*P<0.05, **P<0.01) and group (A) BSA (+P<0.05, ++P<0.01)
Figure 6
Figure 7

Influence of supplemental serum components and FSH on the in vitro germinal vesicle breakdown (GVBD) of bovine follicular oocytes. No significant difference for GVBD among groups, but significant for metaphase II when compared with group (A) BSA (*P<0.05)
Figure 7
Figure 8

Influence of supplemental serum components and HCG on the in vitro germinal vesicle breakdown (GVBD) of bovine follicular oocytes. No significant difference when compared with group (A) BSA.
Figure 8:

The diagram illustrates the percentage of germinal vesicle breakdown in various conditions. The x-axis represents different serum supplements: (A) BSA, (B) FF, (C) ECS, and (D) FCS. The y-axis shows the percentage of metaphase I and II. The bars indicate the proportion of cells in metaphase I and II for each serum supplement.
Influence of supplemental serum components and FSH plus HCG on the in vitro germinal vesicle breakdown (GVBD) of bovine follicular oocytes.

No significant difference for GVBD among groups, but significant for metaphase II when compared with group (A) BSA (*P<0.05, **P<0.01)
Figure 9

Germinal Vesicle Breakdown

<table>
<thead>
<tr>
<th>Serum Supplement</th>
<th>(A) BSA</th>
<th>(B) FF</th>
<th>(C) ECS</th>
<th>(D) FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metaphase I</td>
<td></td>
<td>*</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Metaphase II</td>
<td></td>
<td></td>
<td></td>
<td>**</td>
</tr>
</tbody>
</table>

Figure 9
Figure 10

Supplemental serum components and gonadotropins influence on degenerative frequency of bovine follicular oocytes during in vitro maturation. Significant difference when compared with no gonadotropins (*P<0.05, **P<0.01)
Figure 10
Figure 11

Incidence of sperm penetration of bovine follicular oocytes fertilized in vitro.
Significant difference when compared with no gonadotropins (*P<0.05, **P<0.01)
Serum Supplement

- (1) ECS
- (2) FSH added
- (3) HCG added
- (4) 1μg/ml FSH 2IU/ml HCG added

No gonadotropins
1μg/ml FSH added
2IU/ml HCG added
1μg/ml FSH 2IU/ml HCG added

Figure 11
Figure 12

Incidence of pronucleus formation of bovine follicular oocytes fertilized in vitro.
★Oocytes were cultured with dead sperm for evidence of parthenogenic pronucleus formation.
Significant difference when compared with no gonadotropins (*P<0.05)
Figure 13

Acrosome reaction of bovine spermatozoa cultured in vitro.
*Sperm completely lost acrosome cap (false acrosome reaction)
Figure 13

- Acrosome reaction
- Sperm motility

% 50

0 1.0 2.0 3.0 4.0 h
Figure 14

Protein analysis of follicular fluid (FF), estrous cow serum (ECS), and fetal calf serum (FCS) by gel electrophoresis. *BSA: (1) 0.6 ug, (2) 1.0 ug, (3) 1.6 ug, (4) 2.0 ug, (5) 3.0 ug, (6) 4.0 ug
Figure 14
Figure 15

Bovine oocytes with thick cumulus cell layers collected from the ovary and the zona pellucida (ZP) and oocyte cytoplasm (O) are visible through the cumulus cells. X 40

Figure 16

Bovine oocytes with scattered cumulus cells (CC) from the ovary have undergone atresia, shrunken cytoplasm and condensed chromatin complement. X 40
Figure 17
A bovine oocyte (O) with expanded cumulus cells (CC) cultured for 28 hours. The cumulus expansion is one of significant changes during maturation. X 40

Figure 18
A bovine oocyte with degenerating cumulus cells (CC) cultured for 28 hours. Scattered or condensed cumulus cells are typical characteristics of degenerating oocytes. X 40
Figure 19

A viable oocyte after 28 hours in culture was evenly stained with acetic-orcein and the first polar body (PB) and chromosomes (Chr) are visible. X 80

Figure 20

A degenerating oocyte after 28 hours in culture was stained less and chromatin complement is not visible. X 80
Figure 21

An oocyte is at the germinal vesicle stage. The nucleus (N) appeared to be anchored to the inner surface of the nuclear envelope (NE). X 400

Figure 22

The nuclear envelope (NE) remained and the chromosomes (Chr) were condensed and thickened. This oocyte is classified as at germinal vesicle stage. X 800
Figure 23
Metaphase I of an oocyte after 28 hours in culture demonstrating that the nuclear membrane has broken down and the chromosomes clearly visible in the cytoplasm. X 800

Figure 24
Early anaphase of an oocyte after 28 hours in culture demonstrating the meiotic spindles (S) joining the dividing chromosomes (Chr). X 800
Figure 25

Late anaphase of an oocyte after 28 hours in culture shows that the chromosomes (Chr) segregate into two and migrate to the opposite ends of the spindle (S). X 800

Figure 26

Metaphase II of an oocyte after 28 hours in culture shows that a first polar body (PB) is present within the perivitelline space and the chromosomes (Chr) are identified in the cytoplasm. X 800
Figure 27

An oocyte was examined by electron microscopy after 28 hours in culture. The cumulus cells (CC) adjacent to the zona pellucida (ZP) had cumulus cell projections (CCP) which penetrated it and ended in enlarged perivitelline space (Pvs). X 10,200

Figure 28

An oocyte was examined by electron microscopy after 28 hours in culture. The cortical granules (CG) are closely located to the cytoplasmic membrane and microvilli projections (Mv) developed into the perivitelline space (Pvs). The mitochondria (M) are closely related to the smooth endoplasmic reticulum (SER) and are present in the cytoplasm. X 21,500
A spermatozoon is in the oocyte cytoplasm. The sperm head (SP) is decondensed and enlarged in size and the sperm tail is detached from the head. X 800

Figure 30
A single pronucleus (PN) is formed in the cytoplasm. The second polar body (PB) is closely located to the pronucleus. X 400
Two well developed pronuclei (PN) of an oocyte after 24 hours in co-culture with sperm are in the cytoplasm. The pronuclei are closely associated and are spherical in shape. X 160, X 800
An oocyte after 24 hours in co-culture with sperm was abnormally fertilized and three well developed pronuclei (PN) are in the cytoplasm. X 160, X 800
Figure 35
Sperm were stained with the modified giemsa staining technique for determination of the acrosome reaction. A sperm display dark acrosome (A) which evenly distributed over the anterior region is considered to be "intact". X 800

Figure 36
The acrosome reaction initiates: multiple fusions between the plasma and outer acrosome membranes are visible (indicated by arrow). X 800
Figure 37

The acrosome reaction in progress: more membrane fusions over the head region are visible (indicated by arrow). X 800

Figure 38

The acrosome reaction is completed: the sperm completely lost its acrosome. X 800
Table 1

Influence of supplemental serum components on the in vitro cumulus cell expansion on bovine follicular oocytes

<table>
<thead>
<tr>
<th>Protein suppl.</th>
<th>Expanded cumulus-cell</th>
<th>Non expanded cumulus-cell</th>
<th>Degenerative cumulus-cell</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ham's</td>
<td>10 (13.7)</td>
<td>42 (57.5)</td>
<td>21 (28.8)</td>
<td>73</td>
</tr>
<tr>
<td>BSA</td>
<td>82 (51.6)**</td>
<td>36 (22.6)</td>
<td>41 (25.8)</td>
<td>159</td>
</tr>
<tr>
<td>FF</td>
<td>49 (52.7)**</td>
<td>41 (44.1)</td>
<td>3 (3.2)</td>
<td>93</td>
</tr>
<tr>
<td>ECS</td>
<td>106 (64.2)**+</td>
<td>32 (19.4)</td>
<td>27 (16.4)</td>
<td>165</td>
</tr>
<tr>
<td>FCS</td>
<td>63 (51.2)**</td>
<td>49 (39.8)</td>
<td>11 (9.0)</td>
<td>123</td>
</tr>
</tbody>
</table>

Significant difference when compared with Ham’s F-10 only (*P<0.05, **P<0.01) and Group (A)BSA (+P<0.05)

BSA: 5 mg/ml bovine serum albumin; FF: 10% follicular fluid; ECS: 10% estrous cow serum; 10% fetal calf serum
<table>
<thead>
<tr>
<th>Protein suppl.</th>
<th>Expanded cumulus-cell</th>
<th>Non expanded cumulus-cell</th>
<th>Degenerative cumulus-cell</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>77 (57.0)</td>
<td>51 (37.8)</td>
<td>7 (5.2)</td>
<td>135</td>
</tr>
<tr>
<td>FF</td>
<td>67 (62.0)</td>
<td>39 (36.1)</td>
<td>2 (1.9)</td>
<td>108</td>
</tr>
<tr>
<td>ECS</td>
<td>86 (71.7)*</td>
<td>30 (25.0)</td>
<td>4 (3.3)</td>
<td>120</td>
</tr>
<tr>
<td>FCS</td>
<td>106 (72.6)*</td>
<td>34 (23.3)</td>
<td>6 (4.1)</td>
<td>146</td>
</tr>
</tbody>
</table>

*Significant difference when compared with group (A) BSA (P<0.05).
Table 3

Influence of supplemental serum components and HCG on the in vitro cumulus expansion of bovine follicular oocytes

<table>
<thead>
<tr>
<th>Protein suppl.</th>
<th>Expanded cumulus-cell</th>
<th>Non expanded cumulus-cell</th>
<th>Degenerative cumulus-cell</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>36 (56.3)</td>
<td>23 (35.9)</td>
<td>5 (7.8)</td>
<td>64</td>
</tr>
<tr>
<td>FF</td>
<td>51 (56.0)</td>
<td>39 (42.9)</td>
<td>1 (1.1)</td>
<td>91</td>
</tr>
<tr>
<td>ECS</td>
<td>59 (67.1)</td>
<td>28 (31.8)</td>
<td>1 (1.1)</td>
<td>88</td>
</tr>
<tr>
<td>FCS</td>
<td>58 (71.6)</td>
<td>21 (25.9)</td>
<td>2 (2.5)</td>
<td>81</td>
</tr>
</tbody>
</table>

No significant difference when compared with group (A) BSA (P<0.05)
Table 4

Influence of supplemental serum components and FSH plus HCG on the in vitro cumulus cell expansion of bovine follicular oocytes

<table>
<thead>
<tr>
<th>Protein suppl.</th>
<th>Expanded cumulus-cell</th>
<th>Non expanded cumulus-cell</th>
<th>Degenerative cumulus-cell</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>60 (55.1)</td>
<td>43 (39.4)</td>
<td>6 (5.5)</td>
<td>109</td>
</tr>
<tr>
<td>FF</td>
<td>92 (73.0)**</td>
<td>31 (24.6)</td>
<td>3 (2.4)</td>
<td>126</td>
</tr>
<tr>
<td>ECS</td>
<td>101 (69.2)*</td>
<td>40 (27.4)</td>
<td>5 (3.4)</td>
<td>146</td>
</tr>
<tr>
<td>FCS</td>
<td>114 (78.1)**</td>
<td>27 (18.5)</td>
<td>5 (3.4)</td>
<td>146</td>
</tr>
</tbody>
</table>

Significant difference when compared with group (A) BSA (*P<0.05, **P<0.01).
Table 5

Influence of supplemental serum components on the in vitro germinal breakdown (GVBD) of bovine follicular oocytes

<table>
<thead>
<tr>
<th>Protein suppl.</th>
<th>GV ( % )</th>
<th>M I ( % )</th>
<th>M II ( % )</th>
<th>D ( % )</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>114 (97.4)</td>
<td>1</td>
<td>0 (0.9)</td>
<td>2 (1.7)</td>
<td>117</td>
</tr>
<tr>
<td>Ham's</td>
<td>25 (41.7)</td>
<td>1</td>
<td>17 (30.0)</td>
<td>17 (28.3)</td>
<td>60</td>
</tr>
<tr>
<td>BSA</td>
<td>63 (40.4)</td>
<td>43</td>
<td>16 (37.8)</td>
<td>34 (21.8)</td>
<td>156</td>
</tr>
<tr>
<td>FF</td>
<td>26 (28.0)</td>
<td>19</td>
<td>30 (52.7)**+</td>
<td>18 (19.4)</td>
<td>93</td>
</tr>
<tr>
<td>ECS</td>
<td>19 (15.2)</td>
<td>35</td>
<td>41 (60.8)**++</td>
<td>30 (24.0)</td>
<td>125</td>
</tr>
<tr>
<td>FCS</td>
<td>24 (21.2)</td>
<td>29</td>
<td>50 (70.0)**+++</td>
<td>10 (8.8)</td>
<td>113</td>
</tr>
</tbody>
</table>

Significant difference when compared with Ham's F-10 only (*P<0.05, **P<0.01) and group (A) BSA (+P<0.05, ++P<0.01). GV = germinal vesicle breakdown, M I = metaphase I, M II = metaphase II, D = degenerate.
Table 6

Influence of supplemental serum components and FSH on the in vitro germinal vesicle breakdown (GVBD) of bovine follicular oocytes

<table>
<thead>
<tr>
<th>Protein suppl.</th>
<th>GV</th>
<th>M I</th>
<th>M II</th>
<th>D</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>31 (20.5)</td>
<td>47</td>
<td>51 (64.9)</td>
<td>22 (14.6)</td>
<td>151</td>
</tr>
<tr>
<td>FF</td>
<td>20 (19.8)</td>
<td>21</td>
<td>43 (63.3)</td>
<td>17 (16.8)</td>
<td>101</td>
</tr>
<tr>
<td>ECS</td>
<td>31 (23.5)</td>
<td>30</td>
<td>57 (65.9)</td>
<td>14 (10.6)</td>
<td>132</td>
</tr>
<tr>
<td>FCS</td>
<td>26 (22.8)</td>
<td>23</td>
<td>*54 (67.6)</td>
<td>11 (9.6)</td>
<td>114</td>
</tr>
</tbody>
</table>

No significant difference for GVBD among group, but significant for metaphase II when compared with group (A) BSA (*P<0.05).
Table 7

Influence of supplemental serum components and HCG on the in vitro germinal vesicle breakdown (GVBD) of bovine follicular oocytes

<table>
<thead>
<tr>
<th>Protein suppl.</th>
<th>GV</th>
<th>M I</th>
<th>M II</th>
<th>D</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>26 (41.9)</td>
<td>8</td>
<td>25 (53.3)</td>
<td>3 (4.8)</td>
<td>62</td>
</tr>
<tr>
<td>FF</td>
<td>34 (41.0)</td>
<td>9</td>
<td>34 (51.8)</td>
<td>6 (7.2)</td>
<td>83</td>
</tr>
<tr>
<td>ECS</td>
<td>23 (26.7)</td>
<td>20</td>
<td>36 (65.2)</td>
<td>7 (8.1)</td>
<td>86</td>
</tr>
<tr>
<td>FCS</td>
<td>24 (31.2)</td>
<td>20</td>
<td>31 (66.2)</td>
<td>2 (2.6)</td>
<td>77</td>
</tr>
</tbody>
</table>

No significant difference when compared with group (A) BSA.
Table 8

Influence of supplemental serum components and FSH plus HCG on the in vitro germinal vesicle breakdown (GVBD) of bovine follicular oocytes

<table>
<thead>
<tr>
<th>Protein suppl.</th>
<th>GV</th>
<th>M I</th>
<th>M II</th>
<th>D</th>
<th>Total no. of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>26 (25.5)</td>
<td>39</td>
<td>25 (62.7)</td>
<td>12 (11.8)</td>
<td>102</td>
</tr>
<tr>
<td>FF</td>
<td>30 (24.2)</td>
<td>29</td>
<td>*49 (63.0)</td>
<td>16 (12.9)</td>
<td>124</td>
</tr>
<tr>
<td>ECS</td>
<td>29 (22.7)</td>
<td>36</td>
<td>**53 (69.5)</td>
<td>10 (7.8)</td>
<td>128</td>
</tr>
<tr>
<td>FCS</td>
<td>29 (24.8)</td>
<td>19</td>
<td>**61 (68.4)</td>
<td>8 (6.8)</td>
<td>117</td>
</tr>
</tbody>
</table>

No significant difference for GVBD among groups, but significant for metaphase II when compared with group (A) BSA (*P<0.05, **P<0.01).
Table 9

Influence of Estrous Cow Serum (ECS) and gonadotropins on bovine follicular oocyte fertilized in vitro

<table>
<thead>
<tr>
<th>Gonado. suppl.</th>
<th>2 PN</th>
<th>1 PN</th>
<th>P</th>
<th>S</th>
<th>D</th>
<th>N</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>43</td>
<td>45</td>
</tr>
<tr>
<td>ECS only</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>61</td>
<td>83</td>
</tr>
<tr>
<td>FSH</td>
<td>10</td>
<td>21</td>
<td>28</td>
<td>12</td>
<td>13</td>
<td>80</td>
<td>164</td>
</tr>
<tr>
<td>HCG</td>
<td>6</td>
<td>5</td>
<td>12</td>
<td>5</td>
<td>2</td>
<td>43</td>
<td>73</td>
</tr>
<tr>
<td>FSH+HCG</td>
<td>8</td>
<td>28</td>
<td>31</td>
<td>0</td>
<td>15</td>
<td>32</td>
<td>114</td>
</tr>
</tbody>
</table>

2 PN = Two pronuclei with or without sperm tail remnants, 1 PN = A single pronucleus in the oocyte cytoplasm, P = Polyspermy: more than two sperm heads or more than three pronuclei in the oocyte cytoplasm, S = Sperm in the oocyte cytoplasm without pronucleus development, D = Degenerate: fragmented or scattered chromatin complement in the oocyte cytoplasm, N = No evidence of fertilization.

*Oocytes were cultured with dead sperm for the incidence of parthenogenetic pronucleus formation.
Table 10

Influence of Fetal Calf Serum (FCS) and gonadotropins on bovine follicular oocyte fertilized in vitro.

<table>
<thead>
<tr>
<th>Gonado. suppl.</th>
<th>2 PN</th>
<th>1 PN</th>
<th>P</th>
<th>S</th>
<th>D</th>
<th>N</th>
<th>Total no. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>40</td>
<td>44</td>
</tr>
<tr>
<td>FCS only</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>45</td>
<td>63</td>
</tr>
<tr>
<td>FSH</td>
<td>9</td>
<td>15</td>
<td>16</td>
<td>4</td>
<td>7</td>
<td>60</td>
<td>111</td>
</tr>
<tr>
<td>HCG</td>
<td>5</td>
<td>11</td>
<td>10</td>
<td>4</td>
<td>2</td>
<td>40</td>
<td>72</td>
</tr>
<tr>
<td>FSH+HCG</td>
<td>8</td>
<td>23</td>
<td>6</td>
<td>3</td>
<td>11</td>
<td>63</td>
<td>114</td>
</tr>
</tbody>
</table>

*Oocytes were cultured with dead sperm for the evidence of parthenogenetic pronucleus formation.
Table 11

Acrosome reaction of bovine spermatozoa cultured in vitro

<table>
<thead>
<tr>
<th>Time</th>
<th>Intact acrosome sperm (1)</th>
<th>Acrosome reacted sperm (2)</th>
<th>(3)</th>
<th>(4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>96.4%</td>
<td>0</td>
<td>0</td>
<td>(3.6)*</td>
</tr>
<tr>
<td>1 h</td>
<td>75.7</td>
<td>19.0</td>
<td>4.3</td>
<td>1.0</td>
</tr>
<tr>
<td>2 h</td>
<td>67.7</td>
<td>21.3</td>
<td>7.0</td>
<td>4.0</td>
</tr>
<tr>
<td>3 h</td>
<td>33.3</td>
<td>29.7</td>
<td>26.0</td>
<td>11.0</td>
</tr>
<tr>
<td>4 h</td>
<td>17.6</td>
<td>34.1</td>
<td>36.2</td>
<td>12.1</td>
</tr>
</tbody>
</table>

*Sperm completely lost acrosome (false acrosome reaction). (2): multiple fusion between the plasma membrane and outer acrosome membrane; (3): the reaction is progress; more membrane fusions; (4): complete acrosome reaction
Table 12

Protein analysis

<table>
<thead>
<tr>
<th></th>
<th>T.P</th>
<th>T.Ab</th>
<th>T.P in medium</th>
<th>Ab in medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. BSA</td>
<td>---</td>
<td>---</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>B. FF</td>
<td>81.6+1.36</td>
<td>24.2+0.890</td>
<td>8.2</td>
<td>2.4</td>
</tr>
<tr>
<td>C. ECS</td>
<td>94.1+2.00</td>
<td>33.7+0.582</td>
<td>9.4</td>
<td>3.4</td>
</tr>
<tr>
<td>D. FCS</td>
<td>50.2+0.42</td>
<td>25.1+0.352</td>
<td>5.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*T.P=total protein mg/ml; Ab=albumin; T.Ab=total albumin mg/ml
CHAPTER V

DISCUSSION

The present studies were performed to demonstrate the effects of serum components and gonadotropins on the completion of bovine oocyte in vitro maturation and fertilization.

Maturation of oocytes in vitro

As a control group, 97.4% of oocytes examined immediately after removal from follicle were in the germinal vesicle stage. Similar observations were reported by Jagiello et al. (1974) and this indicated that oocytes from small follicles at any stage of the cycle could be used as a model of oocyte maturation in vitro.

Over 50% of the oocytes cultured in the medium supplemented with serum had cumulus cell expansion while only 13.7% of the oocytes cultured in nonserum-supplemented medium were observed similarly. The results show a significant lack of cumulus expansion of oocytes cultured in the medium with no serum supplement and indicated that serum supplement plays an important active role in supporting the cumulus expansion.
When FSH was added to the medium, ECS and FCS were shown superior to BSA for supporting cumulus cell expansion. Leibfried-Rutledge et al. (1986) suggested that a substance in FCS needed for FSH-induced cumulus expansion was a large molecular weight since a low-molecular-weight filtrate of FCS did not support cumulus expansion. The presence of some unknown materials in serum may play a physiological role in preparing cumulus expansion and it was not a steroid since charcoal-extracted FCS allowed cumulus expansion. It was also predicted that ECS contains unknown metabolic substances which may support cumulus expansion.

Although no significant improvement of any gonadotropins on cumulus expansion was observed in group C (ECS), estrous cow serum induced high percentage of cumulus expansion. This suggests that ECS itself may support cumulus expansion.

A combined effect of FSH plus HCG was tested when both hormones were added to the medium. Only group B (FF) had improved cumulus expansion when compared between the medium with FSH plus HCG and the medium with HCG. In group D (FCS), there was no significant difference between the medium with FSH plus HCG and the medium with FSH or HCG. The addition of any gonadotropins improved cumulus expansion significantly compared with no gonadotropins in group D (FCS). This indicated that the medium supplemented with FCS, FSH or HCG is capable of improving cumulus expansion.
The series of nuclear transformation from germinal vesicle breakdown (GVBD) to the formation of the second meiotic metaphase are evaluated as nuclear maturation of the oocyte. The medium supplemented with FF, ECS, or FCS supported germinal vesicle breakdown effectively compared with Ham's F-10 alone. It was an unexpected observation that there was no significant difference in GVBD between oocytes cultured with or without BSA. The results showed that FF, ECS, and FCS as a serum supplement were superior to BSA. The results suggested that the addition of serum to the medium during oocyte maturation is responsible, at least in part, for the induction of maturation and the GVBD of oocytes was highly dependent on some components which are not included in albumin. The frequency of GVBD was greater in FCS than in BSA as had previously been demonstrated in oocytes from mice (Cross and Brinster, 1970) and bovine (Leibfried-Rutledge et al., 1986). The results of this study agree with the observations by Leibfried-Rutledge et al. (1986) who concluded that FCS was a superior supplement compared with the BSA for in vitro maturation of bovine oocytes.

Some improved frequencies of GVBD by the addition of gonadotropins to the medium were observed in group B (FF), C (ECS) and D (FCS) but there were not significant in this study. The role of gonadotropins in the maturation of the oocyte has been widely acknowledged and experimental
evidence of resumption of meiosis were presented when gonadotropins were added to the medium (Thibaut, 1977; Tsafriri and Channing, 1975). Only group A (BSA) was confirmed the effect of gonadotropins on the GVBD of bovine oocytes.

BSA alone was unable to support viability and maturation of the oocytes during the meiotic division. The inadequacy of the BSA to support the completion of GVBD was evident and suggested that it may not be the best supplement for bovine oocyte culture; however, whether or not the progress of oocyte maturation in the medium with BSA was slow compared with those in the medium with other serum it was not undertaken in this study. BSA constituents need additional evaluation to determine their role in the oocyte maturation.

The effect of bovine follicular fluid as a supplement on oocyte maturation was conducted in this study. The inhibitory activity of follicular fluid from small follicles on the spontaneous maturation of oocytes has been recognized in the pig. In contrast, bovine follicular fluid was ineffective in preventing resumption of meiosis (Sreenan, 1970) and no effect on oocyte maturation was observed (Leibfreid and First, 1980). The follicular fluid from large follicles was expected to contain some factors for promoting oocyte maturation; however, the present study did not verify the effect of bovine follicular fluid on the GVBD
During the oocyte maturation period, both components of the cumulus and oocyte undergo significant morphological and physiological alterations. Morphological and functional transformation of the cumulus cells are inseparable parts of meiotic maturation. The preovulatory maturation of the oocyte comprises nuclear as well as cytoplasmic changes. The meaning of the term "oocyte maturation" includes parallel occurring cytoplasmic events. These processes have been monitored by ultrastructural analysis. Kruip et al. (1983) demonstrated that the cytoplasmic oocyte maturation in vitro, as indicated by the ultrastructural changes, basically followed the same pattern as under in vivo conditions. After the LH peak a series of ultrastructural changes in the oocyte occurred parallel to the nuclear maturation. In the present study, observations by an electron microscope showed some cytoplasmic changes of oocytes after 28 hours in culture. The cumulus cell projections traversed the zona pellucida and ended in the perivitelline space. Hyttel et al. (1986) also observed that perivitelline space developed and enlarged until 9 hours of culture and it was maintained until 40 hours and from 18 hours of culture enlarged cumulus cell projection were frequently present in the perivitelline space.

The oocyte after 28 hours in culture showed that the cortical granules were lying close to the plasma membrane.
In vivo, the cortical granules were found a row closer to the cytoplasmic membrane at 21 to 22 hours after the LH peak (Kruip et al., 1983). This arrangement of granules appears to be a common feature during the late stage of oocyte maturation. Gran and Cheng (1985) showed that the migration of cortical granules which took place between 20 and 30 hours post HCG corresponds to the time of completion of GVBD in the pig. The cortical granule spread is regarded as being essential for oocyte maturation (Sathananthan and Trounson, 1982). In the cytoplasm, the mitochondria closely related to the smooth endoplasmic reticulum are present. Hyttel et al (1986) had the same observation that the mitochondria migrated from their peripheral location to an even spatial distribution in the cytoplasm from 12 to 18 hours of culture and the smooth endoplasmic reticulum formed a large aggregate close to the mitochondria. These ultrastructural findings indicated that nuclear oocyte maturation and the sequence of structural changes which is part of the cytoplasmic maturation are simulated in vitro.

**Fertilization of oocytes in vitro**

As serum supplement, ECS or FCS were added to the medium for fertilization. Sperm penetration rate was higher with ECS plus FSH and HCG but an interaction with gonadotropins led to increased polyspermy. However, the
percent polyspermy frequencies were low compared with the finding of those in other in-vitro fertilization studies in the cow (Leibfried-Rutledge et al., 1986). Others have concluded that FCS did not show any beneficial effects for bovine fertilization in vitro because of the problem of polyspermy. The present study demonstrated that the addition of estrous cow serum and FSH plus HCG to the oocyte maturation medium contributed to the high incidence of pronucleus formation. Although no statistically significant difference between ECS and FCS supplemented with FSH plus HCG was observed in maturation it appears that the presence of FSH plus HCG in the medium might be effective for oocyte maturation and subsequent fertilization. A difference in component between two types of serum may have caused the different fertilization rate. The oocyte may need to obtain specific kinds of substance at the particular time corresponding to the natural process of maturation and fertilization (Sanbuissho and Threlfall, 1985). Metabolic substances in the maternal serum may be transferred to the oocyte during this period (Heller and Schultz, 1980). It is predicted that serum obtained from the cow at the time of estrus contains these substances which contribute to successful fertilization in vitro.

The concentration of each protein in the medium was 9.4 mg/ml ECS and 5.0 mg/ml FCS. Total protein levels in the oviductal fluid near the time of ovulation have been
reported to be between 3 and 14 mg/ml in the pig and sheep (Iritani et al., 1969, 1974) and 4 and 5 mg/ml in the cow (Stanke et al., 1974). This range is low compared with blood serum protein (60-80 mg/ml) but comparable to the presently used levels of protein in medium. The present study did not attempt to determine whether or not higher protein concentrations of ECS rather than its components have any specific role in fertilization.

Parthenogenic development has been observed in the bovine oocyte in vitro (Xu et al., 1986). In this study, the oocytes were cultured in the medium with ECS or FCS for maturation and inseminated with dead spermatozoa for evidence of parthenogenic development. Of 45 (ECS) and 44 (FCS) oocytes, 2 and 1, respectively developed pronucleus. This was low incidence of activation compared with the observations by Xu et al. (1986).

For sperm capacitation and acrosome reaction, bovine follicular fluid (BFF) was used in this study. BFF appeared to stimulated sperm for inducing acrosome reaction in vitro (Yanagimachi, 1969a). A precise role for BFF in stimulating capacitation and acrosome reaction of spermatozoa has not been demonstrated; however, it has been reported that frozen-thawed bull sperm cultured with BFF induced a high fertilization rate in vitro. (Fukui et al., 1983). The exact time required for spermatozoa to penetrate the cumulus cell and zona pellucida is still unknown. Xu and Greve (1988)
examined that the early fertilization events in the cow and concluded that the minimum time required for these events was less than 6 hours. Sperm head decondensation occurred within 1 to 2 hours after penetration and 4 to 6 hours was required for the decondensed chromatin to develop into the subsequent pronuclear stage. In the present study, the culture period was terminated 24 hours after the oocytes and sperm were placed in culture and it was enough for the sperm to decondense and develop pronuclei.

Thibault (1975) reported that the cytoplasm of the oocyte matured in vitro failed to develop the normal pronucleus although the other events of the fertilization process took place normally. In early work, Chang (1955) concluded that normal development after in vitro fertilization is possible only with oocytes recovered from graffian follicles 7 hours after mating or HCG injection. The male pronucleus growth factor (MPGF) is only present in the oocyte 6.5 to 7 hours after the gonadotropic ovulatory stimulation. The abnormal male pronucleus formation indicated that a factor appears in the cytoplasm during preovulatory oocyte maturation in the follicle and this factor is not synthesized in vitro (Thibault, 1975). The pattern of protein synthesis in these oocytes may fail to change in some way as it occurs normally in vivo; partial cytoplasmic maturation (Warnes et al., 1977).

Attempts to fertilize in vitro matured oocytes have
resulted in failure because the male pronucleus did not develop after sperm penetration (Thibault, 1977). It was questioned as to whether these oocytes had the potential to develop normal offspring because of the abnormalities observed in in vitro matured oocytes. In vivo matured bovine oocytes, on the other hand, developed normal calves after fertilization in vitro and transfer to a recipient cow (Brackett et al., 1982, 1984; Lambert et al., 1986). It has been concluded that the developmental incompetence of oocytes matured in vitro results from deficient cytoplasmic maturation even though nuclear maturation appears normal (Thibault, 1977).

Although the mechanisms by which sperm nuclear decondensation and pronucleus formation occur are still unknown, oocytes matured in vitro in the current study were capable of forming pronuclei. The developmental potential of in vitro matured oocytes has been demonstrated in the mouse (Schroeder and Eppig, 1984) and in the cow (Newcomb et al., 1978). Recently, pregnancy was reported by Criste et al. (1986) and Lu et al. (1987) after in vitro fertilization of in vitro matured bovine oocytes and using the sheep oviduct as the in vivo culture system. Xu et al. (1987) also proved that bovine oocytes were matured and fertilized in vitro and subsequently developed into a normal pregnancy after transfer into a recipient heifer. This is conclusive proof that oocytes matured in vitro have the potential for
continuing development. Certain factors may be involved in the capability of in vitro matured oocytes for fertilization and further embryonic development. A possible explanation suggested by Lu, et al (1987) and Xu, et al. (1987) is that the use of estrous cow serum for culture medium is a favorable effect on maturation of bovine oocytes in vitro.

From the results of this study, the culture of bovine oocytes under various conditions allows expansion of cumulus cells and maturation in a large proportion of oocytes in vitro. The supplementation of the culture medium with serum components improved maturation of bovine oocytes and reduced the frequency of degeneration. Some improved frequencies of nuclear maturation by the addition of gonadotropins to the medium were observed in group B (FF), C (ECS) and D (FCS) but there were not significant. The incidence of sperm penetration was increased by the addition of gonadotropins in the medium with ECS. The combined effect of FSH plus HCG on the incidence of pronucleus formation was observed in the medium with ECS or FCS. The medium with ECS and gonadotropins promoted oocyte fertilization over that of FCS.

The maturation processes have to be satisfied for the production of fertilizable oocytes with full developmental capacity. There is an obvious need to concentrate research on the improvement of in vitro culture methods for oocyte maturation. There is justification for believing that in
vitro maturation of the bovine oocyte can be successful if specific requirements of the culture environment are fulfilled.
LIST OF REFERENCES


