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In Vitro Maturation, in Vitro Fertilization and Development of Bovine Oocytes.

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IN VITRO MATURATION, IN VITRO FERTILIZATION AND DEVELOPMENT OF BOVINE OOCYTES

A Dissertation

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Louisiana State University and
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requirements for the degree of
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in

The Department of Animal Science

by

Li Zhang
B.S., Beijing Normal University, 1982
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ABSTRACT

To improve the efficiency of the culture system for bovine in vitro fertilization (IVF), several factors affecting the success of in vitro maturation (IVM), IVF and in vitro culture (IVC) of bovine oocytes were studied. A simple and efficient procedure has been developed for IVM/IVF/IVC of bovine follicular oocytes, which consistently produced 45 to 50% morulae and blastocysts during the treatment of over 6,000 oocytes. Ovary holding temperatures of 0°C, 18°C and 25°C were found to affect the rate of maturation, fertilization and cleavage (P<0.05). When ovaries were held at 25°C for 3 h, 20% of the oocytes developed to blastocysts and hatched in vitro, indicating that this temperature is suitable for holding ovaries during transportation. The highest cleavage rate (79%) was obtained when oocytes were matured and fertilized both at 39°C in 5% CO2. Fertilization rate was impaired with a lowered incubation temperature (37°C) and CO2 level (2.5%). Cumulus cells were shown to be important and necessary for IVM and acquisition of competence for full embryonic development. Cumulus cell removal before IVM, before IVF or 7 h after IVF reduced the rates of maturation, fertilization and development at all embryo stages evaluated (P<0.05). Cumulus cell removal at 20 h after fertilization resulted in a rate of development similar to control embryos (P>0.05). Co-culture enhanced the development of IVF-derived embryos. More embryos developed to blastocysts and hatched when co-cultured with bovine cumulus cells (BCC), bovine oviduct epithelial cells (BOEC) and BCC plus BOEC cells than in medium alone (P<0.05). A simple serum-free medium (CZB) supported more IVF-derived early embryos to the morula stage than a complex serum-containing medium (TCM-199).
However, fewer embryos hatched in CZB medium than in serum-containing TCM-199 (P < .05). Among growth factors evaluated, arachidonic acid (AA) had a significant stimulatory effect on embryo development (P < .05). The optimal AA concentration was 50 ng/ml; a high concentration (500 ng/ml) was toxic. Bovine IVM/IVF/IVC embryos were successfully cryopreserved, thawed and transferred into recipient animals. Nine clinical pregnancies were established with the birth of five normal calves. This success indicates that viable bovine embryos can be produced in the laboratory.
CHAPTER 1

INTRODUCTION

*In vitro* fertilization (IVF) is a controlled process by which oocytes and sperm cells interact with each other under culture condition to produce embryos. In most mammals, fertilization occurs in the ampullar region of the oviduct. The resulting zygote continues to develop as it passes along the oviduct and enters the uterus, and remains free within the lumen of the female reproductive tract until implantation. *In vitro* fertilization avoids the influence of the female reproductive tract and allows gamete interaction to occur in culture, which is a potentially useful tool to investigate the physiological requirements and mechanisms for mammalian fertilization and early embryo development.

For many years, efforts have been made to fertilize mammalian oocytes and develop early embryos *in vitro*. Some of the most extensive studies were conducted in 1951 by Austin and Chang. Their two independent studies clearly demonstrated that sperm cells must undergo a process called capacitation, a series of changes that normally occur in the female reproductive tract, to achieve the capacity to penetrate an oocyte (Austin, 1951; Chang, 1951). In 1959, Chang reported the first birth of mammalian live young after *in vitro* fertilization in the rabbit. In his study, *in vivo*-matured oocytes (ovulated) and *in vivo*-capacitated sperm cells were used to produce transferable embryos.

Thereafter studies on the physiological, endocrinological and biochemical aspects of oocyte maturation and sperm capacitation, as well as early embryo development
extended our knowledge for developing an in vitro system for mammalian fertilization. Successful in vitro fertilizations producing early pregnancies and live births have been reported in many species, including the mouse (Whittingham, 1968), rat (Toyoda and Chang, 1974), hamster (Yanagimachi and Chang, 1964), cat (Pope et al., 1994), pig (Cheng et al., 1986; Mattioli et al., 1989), cow (Brackett et al., 1982a), sheep (Cheng et al., 1986; Crozet et al., 1987), goat (Hanada, 1985a), horse (Palmer et al., 1990), buffalo (Suzuki et al., 1992), monkey (Kuehl and Dukelow, 1979; Bavister et al., 1983) and human (Steptoe and Edwards, 1978). As research continued, in vitro culture systems became more effective; follicular oocytes could be matured in vitro, ejaculated sperm were successfully capacitated in vitro and preimplantation embryos were routinely produced in the laboratory for research purposes, or for embryo transfer to obtain live young. The most success in applying this IVF technology has been in humans. Today, in vitro fertilization has become an effective and widely used method of helping infertile couples conceive children.

Studies of bovine in vitro fertilization were begun in 1970's. Early studies achieved fertilization by inseminating follicular oocytes or ovulated oocytes in the oviduct of a recipient animal or surrogate female (Sreenan, 1970; Hunter et al., 1972; Trounson et al., 1977). The first successful in vitro fertilization in cattle was reported by Iritani and Niwa (1977). In their study, 21 of 103 oocytes were fertilized in vitro with in vivo capacitated sperm cells. However, none of the oocytes were fertilized with in vitro capacitated sperm. Brackett et al. (1978) reported that fertilization occurred in 14 of 25 follicular or ovulated bovine oocytes using sperm cells capacitated in vitro with a high
ionic strength solution. This study demonstrated that bovine sperm capacitation can be induced in vitro. This was the first a repeatable protocol reported for in vitro fertilization in cattle. In 1982, this same group (Brackett et al., 1982a) reported the first calf born as a result of in vitro fertilization of an ovulated oocyte and surgical embryo transfer. Subsequently, Lambert et al. (1983) reported the birth of six calves after transfer of in vitro fertilized embryos produced from laparoscopically recovered oocytes from preovulatory follicles, and cultured in rabbit oviducts.

Later, Hanada et al. (1986), Critser et al. (1986) and Xu et al. (1987) reported live young born after successful in vitro fertilization of in vitro matured (IVM) bovine oocytes. In those studies, surrogate animal oviducts were used for early embryo development. The first IVF calves produced by nonsurgical transfer of IVM, IVF and in vitro cultured (IVC) bovine embryos were reported by Lu et al. in 1987, and soon after by Goto et al. (1988). Co-culture systems were used in their studies and IVF-derived embryos were developed to morula or blastocyst stages in vitro before being transferred nonsurgically to recipient females. Pregnancies and live births have also been reported after transfer of frozen-thawed IVM/IVF/IVC embryos (Fukuda et al., 1990; Kajihara et al., 1992; Zhang et al., 1993). The use of frozen-thawed IVF-derived embryos eliminates the need for synchronizing recipient animals, giving more flexibility to the reproductive management system.

Among farm animals, cattle have become the best studied species for in vitro fertilization. Cattle have been used as a model animal to study follicular growth, oocyte maturation, sperm capacitation, sperm-oocyte interaction and fertilization, and nutrient
and growth factor requirements for early embryo development. Cattle have also been used to study maternal-embryonic genomic transition, pregnancy recognition and in bioengineering studies including embryo sexing, animal cloning and the production of transgenic animals. One advantage of using the cow as a model to study in vitro fertilization is the ready availability of both sperm cells and oocytes. Follicular oocytes can be easily obtained from the ovaries of slaughterhouse animals, and fresh semen can be collected from bulls or cryopreserved for IVF. Within recent years, thousands of articles have been published on bovine IVF and embryo related studies. Several novel protocols for bovine IVF have now been reported (see review by Gordon, 1994). Several laboratories now are able to produce IVM/IVF/IVC bovine embryos for research purposes and for commercial transplantation. However, the efficiency of current IVM/IVF/IVC systems is still low, and the mechanisms underlying in vitro fertilization and early mammalian development are far from being fully understood.

The objective of this study was to improve the efficiency of IVM/IVF/IVC of bovine oocytes by studying several factors that may affect the success rates. A simple procedure described in Chapter 3 was developed to produce a large number of IVM/IVF/IVC bovine embryos from follicular oocytes obtained from abattoir ovaries. Based on this procedure, a series of factors were studied including: 1) the ovary holding temperature during transportation from a local abattoir to the laboratory and the incubation temperature and CO₂ level during oocyte maturation and fertilization in vitro (Chapter 4); 2) the effect of cumulus cells on IVM/IVF/IVC (Chapter 5); 3) the effects of different culture media and "helper" cells on in vitro development of IVF-derived
bovine embryos (Chapter 6) and, 4) the effects of exogenous growth factors on the
development rates of IVM/IVF/IVC bovine oocytes (Chapter 7). Finally, IVM/IVF/IVC
bovine embryos were cryopreserved and thawed approximately one year after storage in
liquid nitrogen, and transferred non-surgically to recipient females to verify the viability
of these in vitro produced bovine embryos by producing live young (Chapter 8).
LITERATURE REVIEW: 
IN VITRO FERTILIZATION AND DEVELOPMENT OF BOVINE OOCYTES

Oocyte In Vitro Maturation

One of the prerequisites for successful fertilization is the completion of oocyte maturation. In mammalian oogenesis, the meiotic process is initiated during fetal life and arrested shortly before or after birth in the prophase diplotene of the first meiotic division (germinal vesicle or GV stage). By the time a female reaches puberty, a group of follicles (a cohort) start to develop under the influence of gonadotrophins, and one or more growing follicles become dominant follicles (Graafian follicles). Oocyte(s) within dominant follicle(s) resume the meiotic maturation process after a preovulatory luteinizing hormone (LH) surge, GV breakdown (GVBD), release of the first polar body, and are again arrested at metaphase of the second meiotic division (M-II). At this time, oocyte maturation (which also includes the changes in the ooplasm known as cytoplasmic maturation) is completed and ovulation takes place.

The phenomenon that mammalian oocytes can undergo spontaneous meiotic resumption when isolated from their follicles was first demonstrated by Pincus and Enzmann (1935). They reported that rabbit oocytes can be matured in vitro even in a hormone-free medium. This phenomenon was later verified in many other mammalian species, including the cow (Chang, 1955a; Edwards, 1965). In 1970’s, the studies on bovine oocyte in vitro maturation showed that nuclear maturation can be achieved after in vitro culture of follicular oocytes, and that these oocytes could be fertilized in the
oviduct of inseminated cows (Sreenan, 1970; Hunter et al., 1972; Shea et al., 1976; Trounson et al., 1977).

Iritani and Niwa (1977) cultured bovine follicular oocytes in modified Kreb's Ringer bicarbonate medium for 24 hours, and inseminated these oocytes with in vivo capacitated sperm. They observed sperm penetration and pronuclei formation in 20% of the inseminated oocytes. The first birth of calves after in vivo fertilization of in vitro matured oocytes was reported by Newcomb et al. (1978). In their study, oocytes aspirated from small follicles were cultured in Ham's F-10 medium supplemented with 20% estrous cow serum (ECS), 1 µg/ml estradiol 17-β (E₂), 1 IU/ml human chorionic gonadotropin (hCG) for 22 to 24 hours resulting in 72% of cultured oocytes developing to the M-II stage; 23% of oocytes recovered a week later from inseminated recipient cows were fertilized and developed to >8-cell stage. This study further verified the viability of in vitro-matured bovine oocytes.

The selection of follicular oocytes has been shown to be an important factor on the success of in vitro maturation and fertilization. Bovine oocytes are usually aspirated from follicles of the abattoir ovaries using a needle attached to a syringe, or the ovaries of live animals using a needle attached to vacuum pump and guided with ultrasonography or laparoscopy.

Fukui and Sakuma (1980) reported that ovarian activity (presence corpora lutea and/or corpus albicans) and the size of the follicles (≤5 mm, 6 to 10 mm and 11 to 20 mm) had no effect on oocyte in vitro maturation. However, the absence of cumulus cells significantly decreased oocyte maturation rate. They also found that the proportion of
oocytes without cumulus cells was increased approximately 30% in the oocytes collected from large follicles compared with that of the smaller follicles. Shioya et al. (1988) reported significantly lower maturation rates, lower fertilization and cleavage rates, and high degeneration rates from naked oocytes compared with cumulus enclosed oocytes after IVM/IVF. Similar findings were also reported by other research groups (Leibfried et al., 1979, Süss et al., 1988; Sirard and Bilodeau, 1990). These findings suggest that the presence of cumulus cells surrounding oocytes is the best indicator of the ability of oocyte to complete meiosis maturation in vitro. The oocytes without cumulus cells (most from large follicles) might have been collected from atretic follicles, and thus degenerating at the time of collection (de Loos et al., 1991).

Süss et al. (1988) and Sirard et al. (1989) studied the time sequence of nuclear progression of bovine oocytes during in vitro maturation. They reported that during in vitro culture, oocytes that have compacted cumulus cells had a nuclear configuration of GV from 0 to 6.6 hours, GVBD at 6.6 to 8.0 hours, metaphase I (M-I) at 10.3 to 15.4 hours, anaphase I (A-I) at 15.4 to 16.6 hours, telophase I (T-I) at 16.6 to 18.0 hours, and M-II at 18.0 to 24.0 hours. This time sequence paralleled the time sequence reported for in vivo matured oocytes (Hafez and Ishibashi, 1964; Kruip et al., 1983; Hunter, 1980; Hyttel et al., 1986), and by the end of the in vitro culture period (24 hours), ~80% of cultured oocytes had reached M-II stage.

Although studies have shown that nuclear maturation can be completed during in vitro culture, cytoplasmic maturation was of primary concern. Thibault and Gerard (1973) reported that in vitro cultured rabbit oocytes could be penetrated by sperm, but
that the sperm head would not enlarge to form normal male pronuclei. Trounson et al. (1977) found 49% of in vitro-matured oocytes fertilized after transfer to the oviduct of an inseminated cow, few had developed to morulae or blastocysts by 96 hours later. In contrast, 39% of in vivo-matured oocytes developed to blastocysts under the same condition and 13 of 16 blastocysts developed to normal fetuses after embryo transfer. Unfortunately, there is no distinct indicator for cytoplasm maturation in oocytes. Therefore, fertilizability and developmental competence of oocytes are often used to indicate the cytoplasm maturity.

Efforts have been made to improve oocyte cytoplasmic maturation by using different culture media, protein supplements, hormones, growth factors, follicular fluid and co-culture. The selection of the medium for culture of bovine follicular oocytes was based on the results of early embryo culture studies. Although some studies have shown that phosphate-buffered medium (PBS) could support bovine and ovine embryo development in vitro (Moore and Spry, 1972; Trounson et al, 1976), no penetration was detected after insemination when this medium was used for an oocyte maturation culture (Baker and Polge, 1976).

Bicarbonate buffered media are generally used for in vitro maturation of bovine oocytes and in vitro fertilization. Eng et al. (1986) reported that bicarbonate ions are required for normal polar body formation. Bicarbonate buffered medium (TCM-199E; Earle’s salts) was used for pig oocyte maturation culture resulting in twice the percentage of polar body formation as the same medium with phosphate buffers (TCM-199H; Hank’s salts). When Hank’s based medium was supplemented with bicarbonate buffer,
polar body formation was restored to the level in Earle's buffered medium. Several other studies also reported the beneficial effect of bicarbonate buffering systems on oocyte maturation and early embryo development over that of other media (Wales et al., 1969; Quinn and Wales, 1973; Kane, 1975; Rajamahendran et al., 1985).

Both simple defined and complex media have been used for oocyte maturation in previous studies. Iritani and Niwa (1977) used Kreb's Ringer medium and Hunter et al. (1972) used Tyrode's medium to \textit{in vitro} mature bovine oocytes. Normal fertilization was observed after insemination with \textit{in vivo}-capacitated sperm cells or after the transfer to oviducts of inseminated cows. Newcomb et al. (1978) used a complex medium (Ham's F-10) for oocyte maturation culture and obtained the first birth of calves from IVM oocytes. The same medium has also been used to produce IVM/IVF calves and IVM/IVF/IVC calves (Xu et al., 1987, 1992b).

The most commonly used culture medium for bovine oocyte maturation is Tissue Culture Medium-199 (TCM-199). Many laboratories have reported good maturation and fertilization rates, moderate morula and blastocyst formation rates and the establishment of pregnancies after embryo transfer using TCM-199 as culture medium (Lu et al., 1987a; Goto et al., 1988; Brackett et al., 1989; Fukuda et al., 1990; Zhang et al., 1992b). Bavister et al. (1992) compared seven different culture media for oocyte maturation \textit{in vitro} and found that the medium used for \textit{in vitro} maturation can strongly affect the developmental capacity of \textit{in vitro}-produced bovine embryos. In five of seven media (TCM-199, SFRE, MEMα, MEMα+, RPMI:MEMα) tested in their study, the proportion of oocytes that cleaved (76 to 82%), developed to morulae (25 to 32%) and
blastocysts (12 to 19%) were similar. However, cleavage and development to morulae and blastocysts were significantly reduced when Waymouth and Ham's F-12 (50 and 35%, 14 and 3%, 3 and 1%, respectively) were used for *in vitro* maturation of bovine oocytes.

Protein supplements were considered as an essential component in culture media for oocyte *in vitro* maturation. Blood serum and serum albumin are major proteins added to the culture media. It has been reported that the addition of serum to the medium significantly increased the percentage of oocyte maturation over that of medium alone (Sanbuisscho and Threlfall, 1990). Both Hunter et al. (1972) and Iritani and Niwa (1977) used bovine serum albumin (BSA) as a protein supplement and obtained *in vitro* maturation and *in vitro* fertilization. Newcomb et al. (1978) added estrous cow serum (ECS) to culture medium for oocyte *in vitro* maturation and established pregnancy after transfer of *in vitro*-matured oocytes to inseminated cows.

Shea et al. (1976) reported *in vitro* maturation and subsequent fertilization using fetal bovine serum (FBS) as a protein supplement. Leibfried et al. (1986) compared the effect of FBS and BSA on bovine IVM/IVF and found that FBS was superior to BSA in promoting *in vitro* maturation and fertilization of bovine oocytes. Unfortunately, the effective components in serum are undefined. Eppig et al. (1992) reported that although approximately 70% of mouse oocytes underwent maturation in both serum and BSA containing media, fertilization and development to blastocysts were significantly reduced when oocytes were matured in BSA-containing medium compared with serum-containing medium. Using a chymotrypsin digestion test, they found that the zona pellucida of
oocytes matured in BSA-containing medium were four times more resistant than the zona pellucida of oocytes matured in vivo or in FBS-containing medium, which could be one explanation for reduced fertilization rates. While the hardening of the zona pellucidae of oocytes matured in BSA-containing medium could be prevented by addition of fetuin (a glycoprotein component of FBS), the developmental competence of these oocytes was still not as good as those matured in serum-containing medium. This study suggests that serum growth factors may function during oocyte growth and maturation to promote events leading to successful fertilization and subsequently embryogenesis.

Recently, several research groups reported successful in vitro maturation and fertilization of bovine oocytes in serum-free and even protein-free medium. Saeki et al. (1991) cultured bovine oocytes in TCM-199 supplemented with .3% polyvinyl pyrrolidone (PVP) and obtained 93% maturation rate, which was not significantly different from that of TCM-199 with 10% FBS (88%). Oocytes that matured in protein-free medium could also be fertilized (60 to 94%) and developed to blastocysts (27%) in vitro in protein-free medium (Saeki et al., 1993).

Moor and Trounson (1977) reported that the developmental capacity of in vitro-matured sheep oocytes was affected by both hormones and follicular factors. In their study, oocytes removed from follicles resumed meiosis in culture, but did not undergo normal development after transfer to inseminated animals. When oocytes were in vitro matured within follicles, more oocytes that were cultured in hormone-containing medium [follicle stimulating hormone (FSH), LH, estradiol-17β (E2)] developed to blastocysts after transplantation than those cultured in hormone-free medium. Newcomb et al.
(1978) obtained a live birth from in vitro matured and in vivo fertilized bovine oocytes using Ham's F-10 supplemented with estrous cow serum (ECS), gonadotropins and E₂ as the oocyte maturation medium. Many research groups thereafter studied the effect of serum, gonadotropins and steroid hormones on in vitro maturation of bovine oocytes, and the results remain controversial.

Younis et al. (1989) compared different sera and hormone additions during bovine oocyte maturation on subsequent in vitro maturation and in vitro fertilization rates. They found that more oocytes matured when using cow sera (Day-0, Day-1, Day-10 and Day-20) compared with FBS, and more fertilized oocytes developed to 4- to 8-cell stage when Day-20 cow serum was used during in vitro maturation. The addition of hormones (E₂, E₂ + LH, E₂ + FSH) improved IVM results and, when LH or FSH was added with E₂, the development to 4- to 8-cell stage was markedly enhanced.

Brackett et al. (1989) reported that oocytes matured with high LH resulted in embryos of excellent viability. Pregnancies were established after embryo transfer. Their further study (Zuelke and Brackett, 1993) indicated that LH acts via the cumulus cells to increase glutamine metabolism within oocytes and, thus, enhance oocyte quality during the maturation process. Sanbuissho and Threlfall (1990) and Saeki et al. (1991) reported improved pronucleus formation, fertilization, cleavage and blastocyst formation when hormones (LH, FSH, E₂) were presented in in vitro maturation medium. The maturation rates, however, did not differ among treatment groups.

In contrast, Fukui and Ono (1989) reported neither the addition of serum (FBS or ECS) nor hormones affected in vitro maturation, cleavage and blastocyst formation in
cattle. Kruip et al. (1988) reported a negative effect of high concentrations of E₂ on bovine oocyte spindle formation and polar body extrusion. Yang et al. (1993) combined different oocyte maturation and sperm capacitation procedures and found that when Ca²⁺ ionophore was used for sperm capacitation, no differences were detected in cleavage and blastocyst formation between hormones and non-hormone maturation groups. However, when heparin was used for sperm capacitation, more cleaved embryos developed to blastocysts in the hormone-treated group than the hormone-free group. Their findings suggested that the difference in oocyte developmental capability could be due to the age of oocyte at the time of fertilization. Since it has been reported that capacitation of bovine sperm by heparin in vitro normally takes ~4.5 hours (Parrish et al., 1989) and the hormone (FSH, LH and E₂) addition delayed the course of bovine oocyte maturation (Süss et al., 1988). Therefore, when 22 to 24 hours was used as the maturation incubation interval and in vitro insemination took place at the same time, better embryo development rates resulted from those oocytes matured with hormones and inseminated with heparin-capacitated sperm. Oocytes that matured without hormones could be over matured at the time of penetration by heparin-capacitated sperm, and had impaired developmental capability.

Recently, Boediono et al. (1994) compared superovulated cow serum (SCS) with FBS on bovine oocyte maturation, fertilization and development to blastocyst stage in vitro, and reported a significantly higher percentage of embryos developing to the blastocyst stage in the presence of SCS than FBS. However, the results of SCS analysis showed that the improved development was not related to the level of LH and
progesterone in the sera. The serum that had a low concentration of glucose, fatty acids and cholesterol supported cleavage and blastocyst development in vitro.

Sperm Capacitation

The sperm released from the male reproductive tract of most mammals are not immediately fertile. They have to undergo a physiological change that normally occurs in the female reproductive tract, termed "capacitation" (Austin, 1951; Chang, 1951). At the site of fertilization, fertilizing sperm pass through the cumulus cell layers of the oocyte, bind to the zona pellucida, complete the acrosome reaction (AR), penetrate the zona pellucida and fuse with the plasma membrane of the oocyte (Wassarman, 1987; Saling, 1991). It has been reported that only capacitated sperm are able to enter the cumulus complex, while uncapacitated and acrosome-reacted sperm are excluded from this outer extracellular matrix of the oocyte (Cummins and Yanagimachi, 1986).

Unlike the acrosome reaction, which is clearly defined as the multiple fusions between the outer acrosomal membrane and the overlying sperm plasma membrane and the release of acrosome hydrolases, capacitation is a process with little detectable morphological change in the sperm membrane. Capacitated sperm could be reversed to uncapacitated state by introducing seminal plasma, which is called "decapacitation" (Chang, 1957). It has been reported that the decapacitation factor(s)-treated capacitated sperm cannot pass through the corona radiata (Farooqui, 1983). However, the decapacitation factor(s) can be removed by centrifugation (Bedford and Chang, 1962). It is believed that capacitation involves a modification or removal of a substance coating the sperm surface, which leads to a decrease in membrane negative surface charge, an
efflux of membrane cholesterol and an influx of calcium between the plasma and outer acrosome membranes (Langlais and Robert, 1985).

Changes in sperm flagellar and associate swimming trajectories have been reported for capacitated sperm (Yanagimachi, 1969; Yanagimachi, 1981). This movement has been termed "hyperactivation". It is thought that the vigorous thrusting of the flagellum may mechanically aid sperm passage through the oocyte investment (Yanagimachi, 1981). *In vitro* studies demonstrated that altered motility patterns were calcium-dependent and related to fertilizing ability of sperm cells in several different species (Fraser, 1977; Yanagimachi, 1972; Cummins, 1982; Bird et al., 1989). Therefore, the hyperactivity is often used as an indicator of the sperm capacitation process. It has been proposed that hyperactive movement may result from an elevated intracellular calcium level during capacitation, which is controlled by the calcium pump in the mitochondria (Bradley et al., 1979).

The discovery of sperm capacitation greatly stimulated the study of *in vitro* fertilization. Early studies used the female uterus to capacitate sperm and obtained *in vitro* fertilization in rabbits (Chang, 1955b; Bedford, 1969). Barros and Austin (1967) demonstrated that the tubal fluid collected from the oviducts of hamster was capable of inducing sperm capacitation and acrosome reaction. Yanagimachi (1969) used follicular fluid to capacitate hamster sperm *in vitro*. Gwatkin et al. (1972) reported that cumulus cells surrounding the oocytes could induce sperm capacitation.

The factors which induce sperm capacitation in the female genital tract do not appear to be species-specific. It was reported that rabbit sperm could be capacitated in
the uteri of rats and dogs (Hamner and Sojka, 1967) and hamster sperm could be capacitated with bovine follicular fluid (Gwatkin and Andersen, 1969).

One hypothesis of sperm capacitation proposes that neuraminic acid and steroid sulfate that coat the sperm plasma membrane during sperm maturation in the epididymis are removed by the enzymes (neuraminidase, steroid sulfatase and arylsulfatase) in the female reproductive tract, that causes destabilization of the sperm membrane (Farooqui, 1983). However, the exact molecular basis of capacitation still remains to be determined.

Successful in vitro capacitation of sperm in simple, chemically defined culture media were reported in several species in early 1970s (Toyoda, 1971; Yanagimachi, 1972; Yanagimachi et al., 1976). These studies demonstrated that mammalian sperm capacitation could occur in the absence of the female reproductive tract or female tract secretion. However, there appears to be a species difference in the requirement of physicochemical conditions, such as the time of exposure and the type of energy sources, for in vitro sperm capacitation (Chang and Hunter, 1975; Rogers and Yanagimachi, 1975). Although the reason for the differences remain unknown, it is likely due to the chemical characteristics of the sperm plasma membrane (Yanagimachi, 1977).

The early attempts at bovine in vitro fertilization with in vitro-capacitated sperm were relatively unsuccessful (Sreenan, 1970; Baker and Polge, 1976; Iritani and Niwa, 1977). Failed fertilization with in vitro-capacitated sperm suggested that the seminal plasma component(s) coating the bovine sperm was very stable, and could not be
removed by simple washing and incubating in culture medium like those for mouse and human sperm capacitation.

Brackett et al. (1978) used a high ionic strength solution (380 mOsm) to induce bovine sperm capacitation for \textit{in vitro} insemination resulting in normal fertilization and subsequently 2- to 4-cell development \textit{in vitro}. In 1982, the same group reported the first live young birth from bovine IVF using the high ionic strength medium treated sperm (Brackett et al., 1982a). The latter study along with those of others (Oliphant and Brackett, 1973; Koehler, 1976; Kinsey and Koehler, 1978) suggested that the treatment of sperm with hypertonic medium resulted in the loss or removal of antigens over the acrosomal region of sperm head.

Iritani et al. (1984) reported fertilization of \textit{in vitro}-matured bovine oocytes with sperm capacitated in a chemically defined isotonic medium. In their study, sperm were collected 1 day before insemination, kept for 14 to 18 hours at 20°C in a test tube, preincubated in m-KRB solution for 8 hours, and 58% of IVM oocytes were fertilized after insemination. A similar method using long incubation times has also been used to capacitate bovine sperm by Wheeler and Seidel (1987).

First and Parrish (1988) developed a method using heparin for inducing bovine sperm capacitation. Their group systematically studied bovine tubal fluid and found that estrual cow oviduct fluid contains a capacitating factor. The same factor, however, was not found to be present at the luteal phase of the estrous cycle. They further found the active factor was heat stable, unaffected by protease digestion, precipitated by ethanol, and inactivated by nitrous acid, a reagent that degrades heparin-like glycosaminoglycans.
Their *in vitro* study demonstrated that heparin was effective in inducing bovine sperm capacitation in a dose and time dependent manner. Bovine sperm were capacitated with heparin by 4 hours of incubation, with the maximal effect occurring with 7.5 μg/ml heparin (Parrish et al., 1988). They found that heparin must bind to capacitating sperm and once capacitation has occurred the heparin could be displaced from sperm without causing decapacitation. They also noted that calcium was required for heparin to capacitate sperm, and when sperm were incubated with heparin an uptake of 45Ca++ occurred. Today, heparin treatment is a well accepted method for inducing bovine sperm capacitation. Pregnancies and live birth have been reported using heparin capacitated sperm for *in vitro* fertilization from several different research groups (Critser et al., 1986; Lu et al., 1987a; Xu et al., 1987).

Caffeine is another reagent that has been used to induce bovine sperm capacitation. It has been reported that caffeine was able to increase motility, fertilizing capability and the ability of the sperm to penetrate the cervical mucus in human (Schoenfeld et al., 1973; Atiken et al., 1983), mouse (Frazer, 1979), and rhesus monkey (Boatman and Bavister, 1984). It is believed that caffeine inhibited intracellular phosphodiesterase activity, which caused the accumulation of cyclic adenosine monophosphate (cAMP) (Cai and Marik, 1989). The elevated cAMP levels likely involved control of the calcium ion flux across the sperm membrane, which in turn increased both motility and velocity of normal sperm (Barkay et al., 1984).

Goto et al. (1988) reported live birth from nonsurgical transfer of IVM/IVF and *in vitro*-cultured (IVC) bovine embryos, using caffeine as a sperm stimulating or
activating agent during capacitation. In this study, frozen-thawed epididymal sperm were preincubated for 2 to 3 hours in Brackett-Oliphant (B-O) medium containing BSA and 2.5 mM caffeine before insemination.

Calcium fluxes through the sperm plasma membrane is a universal event that has been reported during sperm capacitation and the acrosome reaction (Dan, 1956, 1967; Yanagimachi and Usui, 1974). In Ca\(^{++}\)-free media, sperm do not undergo the acrosome reaction (AR), even if they are exposed to an AR-inducing stimuli (Yanagimachi, 1975). Didion and Graves (1989) studied the role of Ca\(^{++}\) on bovine sperm and reported that these sperm could survive in a Ca\(^{++}\)-free medium, but both sperm binding and penetration of the zona pellucida were Ca\(^{++}\) dependent. Since calcium influx has been found consistently during sperm capacitation, efforts have been made to capacitate sperm by inducing Ca\(^{++}\) fluxes. One agent that has been used successfully to induce bovine sperm capacitation and AR is the divalent cation ionophore A23187. Addition of calcium ionophore A23187 to a sperm-medium suspension in the presence of calcium were shown to induce sperm capacitation and AR in mouse (Suarez et al., 1987), pig (Smith et al., 1983), human (Atiken et al., 1984), sheep (Shams-Borhan and Harrison, 1981) and bovine (Byrd, 1981; Takahashi and Hanada, 1984; Bird et al., 1989).

Talbot et al. (1976) proposed that calcium ionophore A23187 can form a lipophilic complex with calcium ion and facilitates its transport across the sperm plasma membrane, resulting in sperm capacitation and acrosome reaction. Shams-Borhan and Harrison (1981) have reported that an ionophore-induced sperm acrosome reaction in the ram was similar to natural acrosome reactions in three features: 1) the induction is
calcium-dependent; 2) no morphological differences were found in sperm; and 3) sperm plasma membrane modification was found only in the acrosomal region. Their study also found that higher concentration of ionophore had a detrimental effect on sperm motility. By adding serum albumin shortly after the addition of the ionophore, however, motility could be preserved, while the acrosome reaction occurred as usual.

Hanada (1985) reported fertilization and cleavage development of in vitro-matured caprine oocytes inseminated with ionophore treated buck sperm. Using .1 or .2 \( \mu M \) calcium ionophore A23187 for 30 or 60 seconds, they obtained a fertilization rate of 78.1\%, which was significantly greater than that of the no ionophore group (29.4\%). Fukuda et al. (1990) inseminated oocytes with calcium ionophore A23187 treated bovine sperm and reported birth of normal calves after transfer IVM/IVF/IVC embryos

**In Vitro Fertilization**

*In vitro* fertilization of bovine oocytes can be obtained by co-incubating matured oocytes and capacitated sperm in a simple chemically-defined medium, termed defined medium (DM), Brackett-Oliphant medium, or Tyrode's albumin lactate pyruvate (TALP) medium. This medium is a modified Tyrode's medium developed by Brackett and Oliphant (1975). The insemination medium is basically the same as the sperm capacitation medium, except without the capacitating agent and with added bovine serum albumin (BSA). The medium contains high bicarbonate content and is adjusted to a pH of 7.8. Incubation of the sperm and oocytes is usually completed under 5% \( CO_2 \) in air (First and Parrish, 1987) or 5% \( CO_2 \), 5% \( O_2 \) and 90% \( N_2 \) (Younis et al., 1989). It has been established that the pH and the bicarbonate ion play an important role during sperm
capacitation, sperm penetration and male pronuclear formation (Ijaz and Hunter, 1989; Boatman and Robbins, 1991b).

Co-incubations are usually conducted in 50 to 100 μl microdroplets with 5 to 25 oocytes/drop in petri dishes covered with mineral oil. The sperm concentration for in vitro fertilization ranges from 1 to 20 x 10^6 motile sperm/ml of medium (Brackett et al., 1982a; Goto et al., 1989), and oocytes are usually co-incubated with sperm for 6 to 24 hours (Parrish et al., 1986; Goto et al., 1989), washed several times to remove extra sperm cells, and then transferred into embryo development culture medium. It is important to remove oocytes from the sperm suspension as soon after penetration as possible because excess dead sperm in the culture system could be toxic to the early stage embryos and, therefore, decrease the viability of cultured embryos (Brackett, 1985). Sperm penetration takes ≈6 hours from the time of insemination in cattle (Xu and Greve, 1988). It should be noted that this also depends on the method used for both oocyte maturation and sperm capacitation (Yang et al., 1993).

The incubation temperature has been shown to be critical to the success of in vitro fertilization in farm animals. Early studies using 37°C for bovine IVF resulted in lower fertilization rates than that of in vivo fertilization (see review by Wright and Bondioli, 1981). Lenz et al. (1983) first suggested that in vitro maturation and fertilization of bovine oocyte are temperature-dependent processes. When bovine oocytes were in vitro matured and fertilized at 39°C, which was the highest fertilization rate of the temperatures evaluated in their study, 58% of oocytes were penetrated by sperm.
Another factor that has been reported to affect the outcome of IVF is the source of the semen. Sperm from different bulls result in different fertilization rates in vitro (Hanada, 1985b; Iritani et al., 1986). This phenomena has also been reported in rabbit (Brackett, 1982b), sheep (Fukui et al., 1988) and goat (Zhang et al., unpublished). In our laboratory, we also found the variation among different ejaculations from the same buck (Zhang et al., unpublished). Therefore, it is important to screen the semen sample individually prior to the onset IVF. Parrish et al. (1986) obtained an acceptable fertilization rate using frozen-thawed bovine semen to inseminate IVM oocytes. Pregnancies resulted with the use of frozen-thawed semen from the same group (Critser et al., 1986). These findings allowed IVF laboratories to decrease the variability often noted from one semen batch to another. Semen from the same bull and same ejaculation can be cryopreserved separately in many straws and tested before its use in IVF.

*In Vitro* Development of IVF-Derived Embryos

The methodology for the *in vitro* development of farm animal IVF embryos is based on the early embryo culture studies in which the embryos were collected from superovulated females. The earliest attempts to culture bovine embryos *in vitro* were reported by Pincus (1951), Brock and Rowson (1952) and Sreenan et al. (1968), but limited development was observed from these early stage embryos. Tervit et al. (1972) reported successful culture of early stage bovine embryos to the morula and blastocyst stages using synthetic oviduct fluid (SOF) medium. Three of five 8-cell bovine embryos developed to blastocysts after being cultured for 6 days, however, all the 1-cell embryos were retarded by completion of the culture interval. Shea et al. (1974) cultured early
stage bovine embryos in BMOC-3 and SOF medium supplemented with HEPES buffer and found that the embryos at 8- to 12-cell stages developed at a higher rate than embryos at an earlier stage of development. When Kanagewa (1979) cultured bovine embryos in BMOC-3 medium, 82% of 8- to 12-cell stage embryos developed into expanded blastocyst after 2 to 4 days of culture. There was no development, however, from 2- to 3-cell stage embryos. These initial findings suggested that there is a 8- to 16-cell stage in vitro developmental block in bovine embryos.

The in vitro developmental block was first described in mouse embryos (Whittingham and Biggers, 1967, 1968). It has been found that mouse embryos from outbred strains do not generally develop into blastocysts when cultured from the 1-cell stage in chemically defined media, but arrest development at the 2-cell stage (2-cell block). However, embryos collected from the same mice at the 2-cell stage or 1-cell stage embryos from inbred strains can develop into normal blastocysts in the same culture medium (Whittingham and Biggers, 1967, 1968; Biggers, 1971; Whittingham, 1974). Studies have shown that the "2-cell block" could be prevented by injecting cytoplasm from embryos of a non-blocking strain (Muggleton-Harris et al., 1982), and "2-cell blocked" mouse embryos could be rescued by transferring to oviducts placed in an organ culture system (Whittingham and Biggers, 1967). These findings suggested that the in vitro developmental block is due to a culture-induced cytoplasmic defect, which does not permit normal development of those mouse embryos beyond 2-cell stage.

In vitro developmental block has also been found in other laboratory and farm animals, including hamster at 2- to 4-cell stage (Whittingham and Bavister, 1974), rat at
4- to 8-cell stage (Bavister, 1988), sheep at 8- to 16-cell stage (Wintenberger et al., 1953), goat at 8- to 16-cell stage (Betteridge, 1977), cow at 8- to 16-cell stage (Thibault, 1966; Camous et al., 1984) and pig at 4- to 8-cell stage (Davis and Day, 1978). These in vitro developmental blocks were found to be related to the maternal-embryonic genome transition in early stage embryos. In many mammalian animals, the earliest stages of embryogenesis are dependent upon stored product of maternal genome (Davison, 1976). Both maternal RNA and protein are accumulated within the oocyte that are sufficient for the progression of the embryo development through the first cleavage division in the mouse (Golbus et al., 1973), the second cleavage division in the rabbit (Van Blerkom and Manes, 1974) and the pig (Schoenbeck et al., 1993) and the third cleavage division in the sheep (Crosby et al., 1988) and the cow (Frei et al., 1986). Subsequent embryonic development is dependent upon the activation of the embryonic genome and the synthesis of embryonic RNA and protein. During this transition, the metabolic requirements of embryo development in some species or strains appear to be difficult to satisfy in vitro.

Efforts have been made to overcome the 8- to 16-cell block during in vitro culture of early stage bovine embryos. Wright et al. (1976b) tested different culture media [minimum essential medium (MEM), TCM-199, BMOC-3, SOF and Whitten's medium] and protein supplements (FBS and BSA). Blastocyst expansion and hatching was achieved from 2- to 8-cell stage embryos when cultured in Ham's F-10 medium supplemented with 10% FBS. No development past early blastocyst stage was achieved when BSA was used as a protein supplement. Although the success rate was low (two of fourteen 1- to 2-cell embryos developed to expanded blastocysts and one hatched), this
was the first report that described complete hatching *in vitro* of bovine embryos cultured from 1- to 2-cell stage.

Since there was no efficient *in vitro* culture system available for bovine embryos prior to the 1980s, early stage IVF-derived embryos were either transferred surgically to the oviduct of recipient cows or transferred into sheep or rabbit oviducts, flushed out 5 to 7 days later to recover morula and blastocyst stage embryos and then transferred nonsurgically into recipient cows.

Brackett et al. (1982a) reported the first IVF live birth in cattle after transfer of an IVF-derived embryo to a recipient cow. In this study, oocytes were recovered surgically from the ovaries prior to ovulation or from oviducts shortly after ovulation and co-incubated with the sperm cells that had been treated with high ionic strength medium (380 mOsm/kg) to induce capacitation. A 4-cell stage IVF-derived embryo was transferred surgically into the oviduct of a recipient cow resulting a normal pregnancy and live birth.

Critser et al. (1986) transferred IVM/IVF 1-cell stage bovine embryos into the oviducts of sheep for cleavage and blastocyst development. Recovered blastocysts were then nonsurgically transferred into recipient cows. Pregnancies and normal development were reported from this study. Lu et al. (1987a) and Xu et al. (1987) used the same approach for developing early stage IVF-derived bovine embryos and reported live birth after nonsurgical embryo transfer. Although the successes of producing live young from IVF-derived embryos were encouraging, these procedures were relatively complicated and time consuming.
Kuzan and Wright (1982) were among the first to use a somatic cell co-culture system to develop bovine embryos to the blastocyst stage *in vitro*. They cultured morula stage embryos with bovine uterine fibroblast cells (BUFC) and bovine testicular fibroblast cells (BTFC) in MEM medium supplemented with 10% FBS, and found that more embryos developed to blastocysts and hatched blastocysts when co-cultured with BUFC than cultured in MEM alone. The beneficial effect of BUFC on the *in vitro* development of bovine morula stage embryos have also reported by other research groups. Voelkel et al. (1985) co-cultured bisected bovine morula stage embryos on a monolayer of BUFC for 72 hours. The embryo viability was significantly improved compared with those demi-embryos cultured with Ham's F-10 medium alone. Wiemer et al. (1987) developed a fetal bovine uterine fibroblasts cell (FBUFC) co-culture system to *in vitro* develop bovine morula stage embryos, and reported enhanced development over that of culturing in medium alone.

Camous et al. (1984) used bovine trophoblastic vesicles to co-culture 1- to 8-cell stage bovine embryos, and reported that 46% of co-cultured embryos developed to morula stage, which was significantly greater than that of embryos cultured in medium alone (18%). Heyman et al. (1987) used trophoblastic vesicle conditioned-medium to culture 1- to 2-cell stage bovine embryos, and 38.8% (14/36) of the embryos developed to the 16-cell stage compared with 41.8% (23/55) of embryos developed to the same stage when directly co-cultured with trophoblastic vesicles. These findings suggested that trophoblast vesicles may produce embryotrophic factor(s) and release the factor(s) into
the culture medium, and this factor(s) is able to enhance the \textit{in vitro} development of bovine embryos beyond the block stage.

Lu et al. (1987b) used a co-culture system to develop IVM/IVF-derived embryos, and reported the first live birth after nonsurgical transfer of IVM/IVF/IVC embryos. In their study, two IVF-derived embryos were co-cultured with bovine oviduct epithelial cells (BOEC) for 6 days before recipient transfer resulting in the birth of twin calves. Goto et al. (1988) also reported live birth from nonsurgical transfer of IVF-derived embryos co-cultured with bovine cumulus cells (BCC).

Within a short period of time, co-culture has become a routine method to \textit{in vitro} develop IVF-derived embryos of the farm animals (see review by Thibodeaux and Godke, 1993). Various types of somatic cells have been found to benefit early stage bovine embryo development, by overcoming 8- to 16-cell \textit{in vitro} developmental block and promoting morula and blastocyst formation.

The cells that have been used for co-culture bovine IVF-derived embryos were from both reproductive and non-reproductive origins. Among reproductive origin cells, BOEC (Lu et al., 1988; Fukui, 1989; Aoyagi et al., 1990; Berg and Brem, 1990; Saeki et al., 1991; Nagao et al., 1991) and BCC (Kajihara et al., 1987; Zhang et al., 1990a; Fukuda et al., 1990; Younis and Brackett, 1990; Iwasaki et al., 1990) are the most extensively used "helper" cells with proved embryo viability by producing live birth after embryo transfer. BOEC monolayers are usually prepared from the abattoir oviducts 48 hours prior to embryo co-culture (Thibodeaux et al., 1992). The BCC monolayers are usually obtained from the cumulus cells that attached to the bottom of the culture dish.
during oocyte *in vitro* maturation incubation and maintained for embryo co-culture (Zhang et al., 1992b).

BOEC cell lines and bovine trophoblast cell lines have also been shown to support bovine IVF embryo development in co-culture. Scodras et al. (1991) co-cultured IVF zygotes with BOEC, BOEC cell line and trophoblast cell line, the percentages of the cultured zygotes that developed to the compacted morula and blastocyst stages (9.7 and 20.9%, 19.4 and 25.8%, 10.1 and 27.6%, respectively) were significantly greater than that of zygotes cultured in medium alone (2.9 and 2.9%). Their results showed that cell lines can support the development of IVF zygotes to morula and blastocyst stages. Another interesting finding from this study was that more compacted morulae resulted from BOEC cell line than freshly prepared BOEC monolayers.

Broussard et al. (1994) used frozen-thawed cumulus cells for co-culture IVF-derived bovine embryos. Cleaved IVF embryos were cultured in medium alone as a control, co-cultured with fresh cumulus cells or co-cultured with frozen-thawed cumulus cells. On day 5 of culture the percentage of embryos that developed to blastocyst stage were 1.5, 20.6 and 23.0%, respectively, and on day 6 of culture the percentage of embryos that developed to blastocysts increased to 3.1, 31.2 and 30.4% respectively. This study demonstrated an improvement of embryo development in co-culture to days 5 and day 6 compared with the control group. However, no differences were found among co-culture groups, which suggested that frozen-thawed cumulus cells can enhance embryo development similar to fresh cells during *in vitro* culture.
Several kinds of non-reproductive origin cells and cell lines have also been used for support IVF embryo development \textit{in vitro}. Kim et al. (1991) reported that bovine fetal spleen (BFS) cells, chick embryo fibroblasts (CEF) cells were able to support the development of early stage IVF-derived bovine embryos to hatched blastocysts. Scodras et al. (1991) used a bovine kidney epithelial cell line (MDBK) and a fetal murine fibroblasts cell line (STO) to co-culture bovine IVF zygotes. Both resulted in improved embryo development over that of medium alone control group.

Voelkel and Hu (1992) used a buffalo rat liver (BRL) cell line to co-culture IVF bovine embryos. When early stage embryos were co-cultured for 6 days, 29\% of the embryos developed beyond compacted morula stage. Those embryos were subsequently frozen and thawed. After thawing, 53\% were viable. Twenty frozen-thawed embryos were transferred into recipient cows resulting in 6 pregnancies (30\%). This study further proved the viability of the bovine IVF embryos that developed on a commercially available cell lines by producing normal pregnancies.

Myers et al. (1994) compared the effect of BOEC, BRL cell line, MDBK cell line and a African green monkey kidney (Vero) cell line on the development of IVF-derived bovine 2- to 4-cell stage embryos. Both monolayer co-culture and conditioned medium culture were evaluated. These results showed that co-culture improved development to the blastocyst stage compared with control medium alone, and the highest development was observed after co-culture with BOEC. Conditioned medium also enhanced development to morulae and blastocysts compared with the control medium; however, no differences were detected among different type of cells.
The supportive effect of somatic cell co-culture on the development of IVF-derived bovine embryos is obvious, however, the mechanism of this effect remains unclear. It has been proposed that the "helper" cells may produce factors that promote embryonic cell proliferation and differentiation and/or metabolize embryotoxic substances from the culture medium (Kane et al., 1992).

Recent Progress in Bovine *In Vitro* Fertilization

The successes of bovine oocyte maturation, sperm capacitation, fertilization and early stage embryo development *in vitro* has greatly stimulated the study of early mammalian development, as the oocytes, zygotes and embryos can be routinely produced in the laboratory using slaughterhouse ovaries. These successes have markedly increased our understanding of the physiologically requirement and regulation of mammalian early development. However, the efficiency of current IVM/IVF and *in vitro* culture systems used to produce viable morula and blastocyst stage embryos from abattoir ovaries is still low, with an estimated average 4.6 blastocysts per animal (Lu and Polge, 1992).

The blastocysts produced *in vitro* had fewer cells, a lower proportion of inner cell mass (Iwasaki et al., 1990), as well as lower post-thaw viability (Rorie et al., 1990; Leibo and Loskutoff, 1993) compared with *in vivo* produced embryos. Recent studies of the metabolism and energy substrate requirements of mouse, rat and hamster embryo development have shown that glucose and phosphate (that normally present in body fluids and contained in the majority of culture medium) can actually be toxic to early stage embryos and/or cause *in vitro* developmental block. In contrast, the addition of glutamine to the culture medium enabled these embryos to overcome the *in vitro* developmental
block (Schini and Bavister, 1988; Chatot et al., 1989; Seshagiri and Bavister, 1991; Miyoshi et al., 1994). Seshagiri and Bavister (1989) reported that phosphate is required for inhibition of development by glucose of hamster 8-cell embryos in vitro. A 2- to 3-fold decrease in oxygen consumption was observed in the embryos cultured with glucose and phosphate (Seshagiri and Bavister, 1991). They proposed that there is a "Crabtree effect" (enhanced glycolysis inhibits respiratory activity and oxidative metabolism) on early stage embryonic cell development, which indicates that the metabolic properties of these embryonic cells differ markedly from those of most somatic cells and resemble some cancer cells. Although glucose seems not to block early bovine embryo development (Pinyopummintr and Bavister, 1991), these findings suggest that there is a potential impact of imbalanced culture medium to cultured embryos.

Kane et al. (1992) suggested that the oocytes and early stage embryos may have enough maternal origin nutrients to support their development before hatching. The reduced developmental competence of in vitro produced and cultured embryos using current culture systems may be explained by the fact that the embryo is swimming in an ocean of fluid into which it is leaking its endogenous nutrients. This is supported by: 1) there is little or no change in protein content of prehatched embryo from 1-cell to morula stage in some of the common mammalian species (Brinster, 1967; Schiffner and Spielmann, 1976; Wright et al., 1981) and 2) the studies on increasing embryo density and reducing the medium volume showed an improvement in embryo ability to overcome the in vitro developmental block and enhancement of morula and blastocyst formation (Paria and Dey, 1990; Nieder and Caprino, 1991; Lane and Gardner, 1992). All of
these findings suggest that the current culture systems are nutrient unbalanced systems for early embryos, and the direction of further studies on the requirement of early mammalian development \textit{in vitro} should be focused on both the addition of nutrient components and the balance of the nutrient components in the culture medium.

The presence of autocrine and paracrine effects of growth factors has also been proposed. The observations that increased embryo density and decreased culture medium volume could enhance embryo development (Paria and Dey, 1990; Nieder and Caprino, 1991; Lane and Gardner, 1992) suggests that certain factors of embryonic origin participated in an autocrine regulation of embryo development. In contrast, the impacted development of \textit{in vitro} cultured embryos compared with \textit{in vivo} produced embryos (Bowman and McLaren, 1970; Iwasaki et al., 1990) indicates that the complement of early stage embryo development requires additional paracrine factors that originate from the reproductive tract.

More progress may be made in the future if growth factors that are involved in early mammalian embryonic development are identified. The expression of growth factor genes (Rappolee et al., 1988), the presence of growth factor receptors (Rappolee et al., 1990; Adamson, 1990), and the growth promoting effect of exogenous growth factors on oocyte and embryo development (Paria and Dey, 1990; Harvey and Kaye, 1992; Das et al., 1991; Yoshida and Kanagawa, 1984; Harper and Brackett, 1993) are now being reported. Using molecular biology techniques, several growth factor ligand and receptor gene expression patterns have been characterized by \textit{in vitro} produced embryos of mouse, sheep and cattle (Rappolee et al., 1988; Watson et al., 1992; 1994). Transcripts
encoding growth factors and growth factor receptors have also been detected in oviductal epithelial cell monolayer cultures, which may explain the beneficial effect of co-culture on early embryo development (Watson et al., 1994). More information is needed to support the hypothesis that growth factors may play an important role in regulation early embryo development.

Our knowledge of early development of mammalian embryos is far from complete. A better understanding of gamete interaction and embryo development from further studies should result in the discovery of culture systems that are more reliable and are more efficiently produce bovine embryos in vitro.
CHAPTER 3

A SIMPLE METHOD FOR IN VITRO MATURATION, IN VITRO FERTILIZATION AND CO-CULTURE OF BOVINE OOCYTES

Introduction

The first calf resulting from in vitro fertilization (IVF) was reported by Brackett et al. in 1982. This feat was duplicated by members of the same research group, with several IVF calves including a set of dizygotic IVF twins, reported in 1984 (Brackett et al., 1984). Since the mid-1980s, IVF technologies have sparked interest (Lu et al., 1987a) and bovine IVF research has thus markedly increased in both the scientific community and in the commercial livestock sector. Key IVF methodologies for bovine oocytes have been reported from different laboratories for early stage in vivo-matured follicular oocytes (Brackett et al., 1982a; Sirard and Lambert, 1986) and from in vitro-matured oocytes harvested from abattoir ovaries (Parrish et al., 1986; Lu et al., 1987a; Xu et al., 1987; Lu et al., 1988; Sirard et al., 1988). Efforts have been made with in vitro culture of fertilized bovine oocytes to overcome 8- to 16-cell in vitro developmental block (Thibault, 1966; Eyestone and First, 1986) to produce IVF embryos at later morphologic stage for nonsurgical transfer to recipient females (Xu et al., 1987; Lu et al., 1988; Sirard et al., 1988). A major barrier remaining in the bovine IVF procedure is to develop an efficient in vitro culture system for the IVF-derived embryos (Lu et al., 1987a; Fukui et al., 1988; Goto et al., 1988).
Transplant pregnancies have resulted from the transfer of IVF-derived bovine embryos using the oviducts of the rabbit (Sirard and Lambert, 1986; Sirard et al., 1985), the sheep (Critser et al., 1986; Lu et al., 1987a; Parrish et al., 1986) and the cow (Lu et al., 1987a; Parrish et al., 1986) as intermediate incubators for developing embryos. More recently, early stage IVF-derived bovine embryos have been cultured to the morula and blastocyst stage *in vitro* using bovine oviduct epithelial cell (Fukui and Ono, 1988; Lu et al., 1988) and the chick embryo amnion (Blakewood and Godke, 1990). Previously, Motlik and Fulka (1981) reported that good success occurs with IVF of rabbit oocytes when incubated with rabbit granulosa cells. More recently, Baird et al. (1990) reported improved *in vitro* embryo development resulted when early stage mouse embryos were co-cultured with granulosa cells harvested from hamsters. Success has also been reported when IVF-derived bovine embryos were *in vitro* cultured with bovine cumulus cells (Goto et al., 1988); however, improvements are needed to increase the efficiency of this procedure. Unfortunately, the basic *in vitro* culture procedures presently in laboratory use are labor intensive, time consuming and generally lack efficiency.

An effort has been made to modify bovine *in vitro* maturation (IVM)-IVF procedures and to combine this approach with a simple, efficient procedure for co-culturing the IVF-derived embryos with the animal's own follicular cumulus cells. This procedure for bovine IVF and co-culture is currently underway in this station and has been used for producing bovine embryos for embryo transfer, freezing, and micromanipulation.
Materials

A. Equipment

1. Laminar-flow hood, Nuaire
2. Temperature-controlled incubator with 5% CO₂, 50°C
3. Temperature-controlled water bath, 50°C
4. Stereomicroscope (10 to 40X), Zeiss
5. Inverted microscope with an UV light attachment (10 to 100X), Nikon
6. Centrifuge, table top, 1000 rpm
7. pH Meter, Microelectrodes
8. Osmometer
9. Hemacytometer, standard, with a 10-μl pipette

B. Chemicals

1. TCM-199 (25 mM HEPES, Earle’s salts), no. 380-2340AG, GIBCO
2. Dulbecco’s phosphate-buffered saline (PBS) (pH 7.2, 270 mOsm), no. 450-1300EB
3. Heat-treated fetal bovine serum (FBS), no. 240-6000AG
4. Brackett-Oliphant medium (B-O medium) (Appendix Table 1)
5. Calcium ionophore (A23187), no. C-7522, Sigma
6. Bovine serum albumen (Fraction V), no. A-4378

1 Nuaire, Minneapolis, MN
2 Zeiss, Thornwood, NY
3 Nikon, New York, NY
4 GIBCO, Grand Island, NY
5 Sigma Chemical, St. Louis, MO
7. Polyvinyl alcohol (PVA), no. P-8136
8. Penicillin-G, potassium, 100 IU/ml, Squibb-Marsam
9. Streptomycin sulfate, 100 µg/ml, Eli Lilly
10. Hoechst stain, no. 33342, no. B-2261
11. 70% Ethanol solution
12. Caffeine-sodium benzoate
13. Mineral oil, medical grade
14. Distilled, deionized water, Milli-Q, 1200 ml

C. Supplies
1. Sterile conical centrifuge tubes, 15 ml, Corning
2. Sterile culture dishes, four-well multidishes, Nunclon
3. Plastic petri dish, 100 mm, Falcon
4. Plastic petri dish, 35 mm
5. Sterile plastic syringe, 5 ml, Becton-Dickenson
6. Sterile needles, 20 gauge
7. Sterile Pasteur glass pipettes
8. Acrodisc syringe filter, 0.22 µm, no. 4192, Gelman

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6 Squibb-Marsam, Cherry Hill, NJ
7 Eli Lilly & Co., Indianapolis, IN
8 Corning, New York, NY
9 Nunclon, Denmark
10 Falcon, New York, NY
11 Becton Dickenson & Co., Lincoln Park, NJ
12 Gelman Sciences, Ann Arbor, MI
9. Sterile glass beakers, 500 ml
10. Adjustable pipettes, 2 to 10 µl, 10 to 100 µl, 100 to 1,000 µl

Procedure

A. Ovary collection and oocyte in vitro maturation

1. Transport the ovaries obtained from heifers and mature cows at an abattoir to the laboratory at temperatures ≥25°C within 3 to 5 hours (h) in sterile Dulbecco’s PBS with antibiotics (100 IU/ml of penicillin-G and 100 µg/ml of streptomycin sulfate).

2. Wipe the bench surface and rinse hands with 70% ethanol.

3. In the laboratory, rinse the ovaries 3 times in Dulbecco’s PBS with penicillin and streptomycin. As a precautionary measure, latex rubber gloves are used when handling the ovaries.

4. Gently dry each ovary with a sterile paper towel.

5. Aspirate 3- to 8-mm diameter follicles at room temperature (22° to 25°C) with a 20-gauge needle attached to 5-ml plastic syringe. Place follicular fluid aspirated from follicles in 15-ml conical centrifuge tubes.

6. After a sedimentation interval (5 min after the last follicle contents are added to the centrifuge tube), aspirate the top layer of follicular fluid leaving 1 to 2 ml of fluid in the bottom of the centrifuge tube.

7. Add 8 ml of PBS with .1% PVA and antibiotics (washing medium) to centrifuge tube, mix and allow oocytes to settle by gravity and then discard
supernatant. Wash the oocytes three times using this procedure, allowing the oocytes to settle via gravity between each washing.

8. After the third washing, resuspend the oocytes in 8 ml of washing medium and place contents into a 100-mm sterile plastic petri dish for oocyte evaluation. Rinse the centrifuge tube twice with the washing medium to recover any remaining oocytes.

9. Transfer oocytes with intact cumulus cells to a 35-mm petri dish and wash twice in washing medium under a laminar flow hood. We recommend not using fluorescent light during this procedure because it may be harmful to oocytes during maturation (Hirao and Yanagimachi, 1978a).

10. Wash cumulus-intact oocytes (20 to 25/group) with .5 ml of room temperature TCM-199 with 5% FBS. Place the oocytes in .5 ml of TCM-199 with 5% FBS (22° to 25°C) in each culture well of a four-well culture plate. Cover each of these culture wells with .5 ml room temperature-equilibrated mineral oil.

11. Incubate the four-well culture plates at 39°C under 5% CO₂ in air for 22 to 24 h.

12. To verify oocyte maturation, cumulus-intact oocytes that did not fertilize after the maturation procedure were exposed to 2% aceto-orcein stain and nuclear material evaluated for metaphase-II morphology. These oocytes were then carefully evaluated for extrusion of the first polar body.
B. Sperm Cell Capacitation

1. Frozen semen from a group of progeny-test dairy and beef bulls (n=5) was evaluated for oocyte IVF capacity and semen from one ejaculate from two of these IVF-compatible bulls and was individually processed and stored in LN₂ for the subsequent IVF procedure.

2. During the IVF procedure, 2 units (e.g., .25 ml plastic semen straw) of semen (from the same bull or two different bulls) were thawed in 25°C water bath for 1 min. Thawing procedure may vary depending on specific recommendations of the organization packaging the frozen semen. In each case, it is recommended to carefully follow the thawing procedure prescribed by the processor.

3. Underneath a sterile hood, the semen straw contents were transferred into a 15-ml conical centrifuge tube.

4. The sperm cells were washed twice with 5 ml of B-O medium (Appendix A) supplemented with 10 mM caffeine sodium benzoate, each time centrifuging at 800 rpm for 6 min.

5. The sperm cell pellet resulting from centrifugation is then resuspended in that volume of B-O medium (usually 2 ml) to adjust the sperm cell concentration to 3 x 10⁶ motile sperm cells/ml.

6. A 20-μM Ca²⁺ ionophore solution was prepared by adding 2.5 ml of Milli-Q water to 50 μl of a 1 mM stock solution of Ca²⁺ ionophore A23187 (Appendix B).
7. The 20-µM Ca++ ionophore solution (10 µl) was added to 2 ml of the resuspended sperm cells (3 x 10⁶ motile sperm cells/ml) for 1 min. The final concentration for sperm cell capacitation medium will then be .1 µM of Ca++ ionophore.

C. In vitro Fertilization

1. After oocyte maturation for 24 h, 20 to 25 cumulus intact oocytes were washed once with B-O medium supplemented with 20 mg/ml of bovine serum albumin (BSA) and then transferred immediately into 50-µl microdrops of the B-O medium with BSA into a 35-mm petri dish.

2. The oocytes were then covered with a 2.5-ml layer of mineral oil (22° to 25°C).

3. A aliquots (50-µl) of capacitated sperm suspension were added to each droplet to give a concentration of 1.5 x 10⁶ motile sperm/ml of B-O medium with BSA.

4. Capacitated sperm cells and cumulus-intact oocytes within the 100 µl fertilization droplets were then incubated at 39° C with 5% CO₂ in an atmosphere of humidified air for 5 h.

D. Embryo Development During In Vitro Co-culture

1. After 5 h of incubation with sperm cells, the oocytes with attached cumulus cells were washed once with .5 ml of fresh TCM-199 supplemented with 5% FBS.
2. The oocytes were covered with a layer mineral oil and then incubated in an atmosphere of 5% CO₂ and air, at 39°C for an additional 43 h.

3. After 48 h of incubation (insemination interval), cumulus cell attached to the embryos were removed by gently teasing with a 30-gauge needle. The cumulus cells were then allowed to remain in the culture wells with the developing embryos.

4. One half of the TCM-199 culture medium was removed at this time and replaced with fresh TCM-199 medium with 5, 10 or 20% FBS to activate the cumulus cell culture system.

5. The culture medium was replaced with fresh medium at 48-h intervals during co-culture. To maximize in vitro development, fresh cumulus cells was added to the culture system at the time the culture medium was replaced. The embryos were examined for development using an inverted microscope (40-100X) at the time the medium was changed.

6. On days 7 and 8 after IVF morulae and blastocysts derived from this procedure were either transferred to recipient females or frozen in liquid nitrogen. Some embryos were stained with the nuclear stain Hoechst stain no. 33342 to aid in assessing cell number during and following co-culture.

Results and Discussion

The experimental results obtained using this modified approach to the in vitro production of bovine morulae and blastocysts are encouraging. Embryos derived from in vitro maturation and in vitro fertilization progressed rapidly through the 8- to 16-cell
in vitro developmental block stage when the embryos were co-cultured with endogenous cumulus cells. The actual result from one recent 30-day trial using the procedure described herein on dairy and beef cows/heifers are presented in Table 1. These results are similar to and, in most cases, better than those reported to date for IVM and IVF-derived embryos from bovine oocytes obtained at slaughter (Fukui and Ono, 1988; Iritani et al., 1984; Lu et al., 1988). This approach to in vitro culture makes the IVF procedure less time consuming, more efficient, and less costly when compared with the other in vivo and in vitro culture methods presently in use at commercial cattle embryo transplant stations.

This approach simplifies many of the procedures reported in the scientific literature that have subsequently become a part of many bovine oocyte in vitro maturation and in vitro fertilization protocols used today. After processing over 6,000 bovine oocytes using this modified procedure in our laboratory, it now seems that the need for adding gonadotropin [e.g., luteinizing hormone (LH) and follicle stimulating hormone (FSH)] and estradiol-17β to the maturation medium, the need for oocyte movement (rocking action) during the maturation procedure, the need to conduct in vitro maturation and fertilization procedures in a heated room (>30°C), and the need for "swim up" and extended capacitation incubation procedures are certainly lessened or possibly eliminated from the standard procedure with the simplified method described herein. With this procedure we normally expect a 90 to 95% maturation rate, an 85 to 90% fertilization rate, a 65 to 75% cleavage rate and 42 to 50% of the IVF-derived embryos to reach the morula stage of development from a random group of bovine oocytes harvested from
Table 1. Results after IVM, IVF and cumulus cell co-culture of bovine oocyte during a 30-day trial

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<tbody>
<tr>
<td>selected to IVM</td>
<td></td>
<td></td>
<td>MATR</td>
<td>FERT</td>
<td>CLEV</td>
<td>MORL</td>
<td>EXBLST</td>
<td>HBLST</td>
</tr>
<tr>
<td>3,212</td>
<td>3,081</td>
<td>3.81</td>
<td>2,927</td>
<td>2,650</td>
<td>2,101</td>
<td>1,350</td>
<td>493</td>
<td>293</td>
</tr>
<tr>
<td>% 96%</td>
<td>–</td>
<td>95%</td>
<td>86%</td>
<td>68%</td>
<td>44%</td>
<td>16%</td>
<td>9.5%</td>
<td></td>
</tr>
</tbody>
</table>

*MATR=Matured; FERT=fertilized; CLEV=cleaved; MORL=morulae; EXBLST=expanded blastocyst; HBLST=hatched blastocyst.

*b Oocytes with tightly compacted cumulus cell layers.

*c Final evaluation and selection for *in vitro* maturation procedure.

*d Cumulus complex expansion score: 1=poor to 4=excellent.
ovaries of healthy, well-nourished, cyclic cows at the time of slaughter (Figures 1 and 2).

The simplified procedure outlined does not call for the addition of supplemental LH, FSH, or estradiol-17β to the maturation medium as previously been proposed (Staigmiller and Moor, 1984), and used routinely by others in bovine (Aoyagi et al., 1988; Shioya et al., 1988), ovine (Fukui et al., 1988), and porcine (Mattioli et al., 1988; Nagoi et al., 1988) oocyte maturation procedures. The approach described herein does include 5% heat-treated FBS in the maturation medium, which could make available to the oocytes small amounts of these hormones during the maturation and culture process. The value of FBS to in vitro maturation and fertilization of bovine oocytes, however, has not been clearly defined. In a recent experiment at this laboratory (unpublished data), 400 bovine oocytes were placed in the TCM-199 with 5% FBS or placed in TCM-199 without FBS. Maturation rates (95.4 vs. 96.5) were similar but significantly increased fertilization and cleavage rates, and a greater percentage of the IVF-derived embryos reaching the blastocyst stage in vitro were found in the system containing FBS compared with the same maturation system without FBS. It is interesting to note that in this study 80% of the oocytes were fertilized and 67% of the oocytes cleaved after oocyte maturation without either supplemental hormones or FBS. Further observations in our laboratory suggest that parthenogenic activation and cleavage (2-cell stage) occurs in up to ≤3% of the bovine IVM oocytes not exposed to sperm cells.

The simplified procedure uses caffeine (Kajihara et al., 1987; Niwa and Ohgoda, 1988) and Ca²⁺ ionophore A23187 to aid in capacitation of the frozen-thawed bovine
Figure 1. Bovine cumulus oocyte complexes (COC) used for producing IVF-derived embryos in this study. Immature cumulus-intact oocytes aspirated from 2 to 8 mm follicles (a). Oocytes that matured for 24 h in TCM-199 with 5% FBS (b). Magnification: X100.
Figure 2. IVF-derived embryos produced from this study. Eight- to 16-cell stage embryos developed 3 days post-insemination with cumulus cell co-culture. Magnification: X200 (a). Expanded blastocysts and hatched blastocysts developed 10 days post-insemination with cumulus cell co-culture. Magnification: X100 (b).
sperm cells similar to that previously reported (Aoyagi et al., 1988; Hanada, 1985; Sugawara et al., 1985). With this method the sperm cells are exposed to the ionophore for only 1 min. This approach was modified from that of an earlier experiment with in vitro capacitation of mammalian sperm cells (Brackett and Oliphant, 1975), and is much less time consuming than the longer, more commonly used methodologies using heparin to enhance capacitation (Leibfried-Rutledge et al., 1987; Parrish et al., 1986).

In a recent experiment in this laboratory (Zhang et al., 1990), the effect of three different ovary holding temperatures during transport from the slaughterhouse to the laboratory were evaluated. When bovine ovaries were cooled on ice at 0°C to 2°C during transport, lower rates of fertilization and embryo development resulted (P < .05) compared with transport temperatures of 18°C or 25°C. This has recently been confirmed by another laboratory making similar temperature comparisons on bovine ovaries (Gordon and Lu, 1990). The reason for this is unclear. When IVM-IVF procedures were conducted at room temperature (22 to 25°C) and at 30 to 32°C in other preliminary experiments in this laboratory, there was no difference in fertilization, cleavage and subsequent embryo development rates. These findings indicate that cooling the bovine ovary before processing may be more detrimental than once expected; however, allowing cumulus-intact oocytes to cool to room temperature during laboratory processing may not be as critical to the overall IVM-IVF procedure as once thought (Lenz et al., 1983).

The results of this study verify the reports of others (Aoyagi et al., 1988; Leibfried-Rutledge et al., 1987; Parrish et al., 1986) in that sperm from different bulls
within the same breed differ greatly in their ability to fertilize ova during the IVF procedure. We have also noted that frozen semen from the same collection gives more repeatable results during bovine IVF than that of different ejaculates from the same bull or from different bulls.

In a recent study in this laboratory (Zhang et al., 1991), cumulus-intact oocytes (n=170) from heifers were compared with cumulus-intact oocytes (n=170) collected from mature cows using the same IVM-IVF procedure. The results from this study showed that oocytes harvested from mature cows gave similar maturation, fertilization and early cleavage rates; however, the percentage of the cleaving embryos reaching the expanded blastocyst and the hatched blastocysts stages \textit{in vitro} was greater (P<.05) for mature cows than of heifers. These observations on parity of the females are in agreement with results of other recent IVF studies (Kajihara et al., 1987, 1991). Possibly, the time interval needed for oocyte maturation is different between mature cows and young heifers (Kajihara,Y, personal communication).

Using the animal's own follicular granulosa/cumulus cells to aid in embryo development to morula-stage embryos has consistently given the better results than of other \textit{in vitro} culture and co-culture systems presently in use in our laboratory. The number of blastomeres per cultured embryo at the morula stage was similar to the number stained and counted for fresh embryos collected from donor cows on the same day post-fertilization. After a basic IVM-IVF procedure with cumulus cell co-culture in our laboratory, 16 good quality morulae were individually transferred to day 7 and 8 recipient cattle, resulting in eight transplant pregnancies (50%) past 90 days and the birth
of seven live, healthy IVF offspring. The current consensus is that development of IVF-derived embryos to the blastocyst stage in vitro suggests that normal fertilization has occurred under laboratory conditions (Staigmiller and Moor, 1984).

Despite the use of various chemically defined media, very little success has resulted with in vitro embryo culture procedures. This has been overcome to some degree by using various cell types in co-culture systems. The use of endogenous cumulus cells and increasing levels of FBS in the culture system certainly has merit and should not be overlooked as a culture system for IVF embryos. The cumulus cell co-culture system is easy to maintain, efficient, and produces results similar to and in most cases better than those reported for other culture or co-culture systems. This approach is cost efficient because the cumulus cells are a by-product of the IVM-IVF procedure.

One drawback with this co-culture system becomes evident when one cultures IVF-derived oocytes to the expanded blastocyst and hatching stages in vitro. When only homologous cumulus cells supplemented with FBS are used in the culture system, half or less of the embryos develop from the morula to the expanded blastocyst stage of development. The reason for this reduced development is unclear. Apparently, other components (growth factors) are needed in the in vitro system that are not present or not available to the embryos in sufficient concentrations during extended incubation with bovine cumulus cells. Furthermore, one can not rule out the possibility of increasing levels of toxic metabolic by-products in the co-culture system hindering further embryonic development at this stage of incubation.
To maximize *in vitro* development of IVF-derived embryos, we suggest adding fresh cumulus cells to the culture system at 48-h intervals during incubation, when the culture medium is exchanged. This may make enough of the unidentified growth factor(s) available to increase the number of embryos developing to the blastocyst stage. Possibly, a multilayered cell culture system of more than one type of cell (e.g., oviductal and cumulus cells) could also be used to maintain the development of these IVF-derived morular to expanded or hatched blastocyst stage (Rodriguez et al., 1991).
CHAPTER 4

EFFECT OF TEMPERATURE AND CO\textsubscript{2} LEVELS ON \textit{IN VITRO} MATURATION AND FERTILIZATION OF BOVINE OOCYTES

Introduction

\textit{In vitro} maturation (IVM), fertilization (IVF) and culture (IVC) of farm animals oocytes can provide a large supply of early stage embryos for basic embryo research, and will allow scientists to study the details of the maturation and fertilization process. At present, although the procedure for IVM/IVF/IVC of bovine oocytes has been established (Brackett et al., 1977; Iritani and Niwa, 1977; Brackett et al., 1982a; Parrish et al., 1986) and calves have been born after nonsurgical transfer of fresh IVM/IVF/IVC embryos (Lu et al., 1988; Goto et al., 1988; Xu, et al., 1992a; Reichenbach et al., 1992) or frozen-thawed IVM/IVF/IVC embryos (Fukuda et al., 1990; Zhang et al., 1993) from abattoir ovaries, the efficiency of the \textit{in vitro} system is relatively low. Since the current IVF procedure is simply an effort to mimic the reproductive tract of the female animal, the physiological factors, such as \textit{in vitro} temperature and CO\textsubscript{2} environment on maintaining oocyte viability, maturation, fertilization and early development are not fully understood.

Cooling intact abattoir ovaries has been reported to be detrimental to oocyte viability (Yang et al., 1990; Smedt et al., 1992). Apparently, the sensitivity of the oocyte to cooling varies among different species (Smith and Tunney, 1978). However, a negative effect on later development of IVF-derived embryos has also been reported.
after bovine ovaries were held at a temperature close to body temperature (37°C) (Yang et al., 1990; Sekin et al., 1992).

Presently, the recommended incubation conditions for bovine IVF is 39°C in 5% CO₂ in air or 5% CO₂, 5% O₂ and 90% N₂ (First and Parrish, 1987; Younis et al., 1989). This incubation temperature is thought to be based on internal core body temperature of the cow (Anderson, 1970). An early research study, using 37°C for bovine IVF, strongly suggested that this temperature lowered the success rate of in vitro fertilization (see review by Wright and Bondioli, 1981). Later, Brackett et al. (1982) obtained the first bovine IVF offspring following fertilization incubation at 38°C. Lenz et al. (1983) subsequently reported that bovine in vitro fertilization was a temperature dependent process and that higher fertilization rates should result with increased temperatures. Grinsted et al. (1980) reported that in vivo follicle temperatures were lower compared with the internal core body temperature of the rabbit by 2.8°C, and that the temperatures were similar for both large and small follicles. The physiological significance of a temperature gradient on follicle growth and oocyte maturation in vivo is presently unclear.

A bicarbonate buffering system has been shown to be beneficial for oocyte maturation and embryo development in mammalian species (Wales et al., 1969; Quinn and Wales, 1974; Kane, 1975; Wright et al., 1976a). Eng et al. (1986) showed that the appropriate level of CO₂ in the gas phase is required not only for maintaining the appropriate medium pH, but is also intimately involved in in vitro oocyte and embryo
metabolism. However, CO₂ levels have not been investigated extensively as a variable that may influence oocyte maturation and *in vitro* fertilization.

The objectives of this study were: 1) to evaluate the effect of different ovary holding temperatures during transportation on the developmental potential of bovine oocytes and 2) to evaluate the effect of incubation temperature and CO₂ levels on bovine oocytes in *in vitro* maturation and fertilization.

**Materials and Methods**

**Experimental design**

**Experiment 4.1. Ovary holding temperature and bovine IVM/IVF/IVC**

The first experiment was designed to evaluate ovary holding temperatures on oocyte maturation, fertilization and subsequent embryo development. In this experiment, three holding temperatures were evaluated. Prior to animal slaughter, three thermos bottles were filled with water at either 25°C, 18°C or 0°C (with ice). Three 50-ml sterile plastic vials containing physiological saline were placed into each thermos. Ovaries from six immature slaughterhouse beef heifers (~6 month old) were immediately placed randomly in one of three 50-ml vials, and placed into the thermos bottles. The bottles were then transported to the St. Gabriel Physiology Laboratory. The elapsed time from ovary collection to oocyte aspiration was 3 h. The ovaries were then subjected to a standard bovine IVF procedure, as outlined in Chapter 3. The experimental outline is shown in Table 2.
Table 2. Experimental design for Experiment 4.1

<table>
<thead>
<tr>
<th>Ovary temperature</th>
<th>No. of ovaries*</th>
<th>No. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°C</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>18°C</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>25°C</td>
<td>4</td>
<td>25</td>
</tr>
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</table>

*Ovaries randomly placed across treatment temperatures.
Experiment 4.2. Incubation temperature, CO₂ levels and bovine oocyte fertilization

The second experiment was designed to evaluate incubation temperatures and CO₂ levels on oocyte maturation and fertilization. Cumulus oocyte complexes (COC) harvested from mixed-breed beef heifers were randomly assigned to one of four treatment groups as follows: 1) oocytes matured at 37°C and fertilized at 37°C in 2.5% CO₂ in air, 2) oocytes matured at 37°C but fertilized at 39°C in 2.5% CO₂ in air, 3) oocytes matured at 39°C and fertilized at 39°C in 2.5% CO₂ in air and 4) oocytes matured at 39°C and fertilized at 39°C in 5% CO₂ in air (standard IVF procedure, as in Chapter 3). The experiment treatments and number of oocytes assigned per treatment is shown in Table 3. After 24 h of maturation, cultured oocytes were inseminated with ionophore A23187-treated sperm cells. The cleavage rate was evaluated 48 h after insemination and used as an indication of successful in vitro fertilization. All cleaved embryos were subsequently cultured at 39°C in 5% CO₂ in humidified air and randomly assigned to another experiment (data not shown here).

Ovary transportation and oocyte collection

Ovaries for these experiment were harvested from cyclic, mixed breed beef heifers at a local abattoir immediately after slaughter and transported at 25°C (or other temperatures as described in specific experiments) to the St. Gabriel Physiology Laboratory in physiological saline (.9% NaCl). In the laboratory, ovaries were rinsed three times in saline containing antibiotics (100 IU penicillin-G, 100 μg streptomycin sulfate/ml) and dried with a sterile paper towel. Oocytes were collected by aspiration of 2- to 8-mm antral follicles using an 18-gauge needle attached to a 6-ml sterile, plastic
Table 3. Experimental design for Experiment 4.2

<table>
<thead>
<tr>
<th>Culture condition*</th>
<th>No. of oocytes</th>
</tr>
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<tbody>
<tr>
<td>37°C/37°C (2.5% CO₂)</td>
<td>600</td>
</tr>
<tr>
<td>37°C/39°C (2.5% CO₂)</td>
<td>600</td>
</tr>
<tr>
<td>39°C/39°C (2.5% CO₂)</td>
<td>600</td>
</tr>
<tr>
<td>39°C/39°C (5% CO₂)</td>
<td>600</td>
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</table>

*Culture condition means the incubation temperature and CO₂ level in each treatment group: maturation temperature/ fertilization temperature (CO₂ level).
syringe. Follicular fluid was placed in 50-ml conical centrifuge tubes. After a sedimentation interval (3 to 5 min.), supernatant was discarded and 10 ml of Dulbecco’s phosphate-buffered saline (PBS, Gibco, Grand Island, NY) with .1% Polyvinyl alcohol (PVA, Sigma, St. Louis, MO) was added to the centrifuge tube. The oocyte-PBS suspension was placed into a 100-mm sterile plastic petri dish for oocyte evaluation. Oocytes with intact cumulus and homogeneous cytoplasm were used in this study. Ovary washing and oocyte collecting were performed at room temperature (23 to 25°C).

In vitro maturation and fertilization

The bovine IVF procedure used was previously developed in this laboratory (Chapter 3). The standard procedure was changed only for temperatures and CO₂ levels as designated by the experimental design. Briefly, selected oocytes were washed two times in PBS with .1% PVA, two times in Tissue Culture Medium-199 (TCM-199; 25 mM HEPES, Earle’s salts, Gibco) supplemented with 5% fetal bovine serum (FBS, HyClone, Logan, UT) and placed in four-well culture plates (Nunclon, Denmark) (20-25 oocytes/well) containing .5 ml of TCM-199 with 5% FBS in each well. Oocytes were incubated 22 h for maturation at 39°C in 5% CO₂ in an atmosphere of humidified air.

Approximately 1 to 2 h prior to insemination, two straws of frozen semen from a pre-selected Holstein dairy bull of known fertility were thawed in a 25°C water bath for 1 min. The sperm cells were washed twice in Brackett-Oliphant (B-O) medium (Brackett and Oliphant, 1975) supplemented with 10 mM caffeine (Niwa and Ohgoda, 1988) and adjusted to a concentration of 3 x 10⁶ motile sperm cells/ml. Sperm cells were then exposed to .1 μM of calcium ionophore A23187 for 1 min to aid in capacitation
(Hanada, 1985; Zhang et al., 1992b). Approximately 50 μl of a sperm suspension was quickly added to each 50 μl fertilization droplet containing oocytes. The fertilization medium was B-O medium supplemented with 20 mg/ml bovine serum albumin (BSA, Fraction-V, Sigma). Oocytes and sperm cells were co-incubated at 39°C in 5% CO₂ in humidified air.

After 6 h of incubation, oocytes with attached cumulus cells were washed with TCM-199 supplemented with 5% FBS and transferred into four-well culture plates containing .5 ml TCM-199 with 5% FBS. Oocytes were cultured for an additional 43 h to evaluate the cleavage rate. The cleaved embryos were subsequently cultured on a cumulus cell monolayer (Goto et al., 1988; Zhang et al., 1992b) at 39°C in 5% CO₂ in humidified air until the embryos reached the hatched blastocyst stage. The culture medium (TCM-199 with 5% FBS) was replaced with fresh medium at 48-h intervals and the embryo development recorded immediately after medium change. All uncleaved oocytes (in Experiment 4.1) were fixed at 48 h post-insemination in ethanol:acetic acid (3:1) for 24 h and stained with 2% aceto-orcein to examine the arrested stages (GV, M-I, M-II and PN).

Statistical analysis

The difference in the proportion of oocytes that matured, fertilized and developed to blastocyst and hatched blastocyst stage among treatment groups were tested using Chi-square analysis for both Experiment 4.1 and Experiment 4.2. A P-value of < .05 was defined as statistically significant.
Results

Experiment 4.1

In this experiment, the effect of three ovary holding temperatures (0°C, 18°C and 25°C) on oocyte maturation, fertilization and subsequent embryo development was evaluated. As shown in Table 4, oocyte maturation was impaired when ovaries were held at 0°C or 18°C for 3 h during transportation compared with 25°C (20%, 58% and 100%, respectively; P < .05). Fertilization rates in 0°C, 18°C and 25°C group were 10%, 54% and 95%, respectively, which was also significantly different among treatment groups (P < .05). There was no cleavage when ovaries were hold at 0°C during transportation although 10% of the oocytes had decondensed sperm heads or showed evidence of two pronuclei. Significantly more oocytes cleaved in the 25°C group (85%) than in the 18°C group (25%) (P < .05). The proportions of the oocytes developing to the morula and blastocyst stages from the 25°C treatment group were also greater than those from the 18°C treatment group (50% vs. 8% and 20% vs. 4%, respectively; P < .05). All blastocysts developed from the 25°C group hatched in vitro; however, none of blastocysts hatched from the 18°C treatment group.

Experiment 4.2

The effect of the combination of two incubation temperatures (37°C and 39°C) during maturation and fertilization and two CO₂ levels (2.5% and 5%) on oocyte fertilization was evaluated in this experiment. The result is shown in Table 5. Only 49 (8%) of the 600 oocytes cleaved when maturation and fertilization temperature were both 37°C and the CO₂ level was 2.5% (37°C/37°C and 2.5% CO₂). The cleavage rate
Table 4. Ovary holding temperature on oocyte maturation, fertilization and subsequent embryo development in Experiment 4.1*

<table>
<thead>
<tr>
<th>Ovary TEMP</th>
<th>No. of oocytes</th>
<th>MATR</th>
<th>FERT</th>
<th>CLEAV</th>
<th>MORL</th>
<th>BLST</th>
<th>HBLST</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°C</td>
<td>20</td>
<td>20(^a)</td>
<td>10(^a)</td>
<td>0(^a)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18°C</td>
<td>24</td>
<td>58(^b)</td>
<td>54(^b)</td>
<td>25(^b)</td>
<td>8(^a)</td>
<td>4(^a)</td>
<td>0(^a)</td>
</tr>
<tr>
<td>25°C</td>
<td>20</td>
<td>100(^b)</td>
<td>95(^c)</td>
<td>85(^c)</td>
<td>50(^b)</td>
<td>20(^b)</td>
<td>20(^b)</td>
</tr>
</tbody>
</table>

*TEMP=temperature; MATR=matured; FERT=fertilized; CLEAV=cleaved; MORL=morula; BLST=blastocyst; HBLST=hatched blastocyst.

*Values with different superscripts within columns are different (P < .05).
Table 5. Incubation temperature and CO₂ level on bovine oocyte fertilization

<table>
<thead>
<tr>
<th>Culture condition*</th>
<th>No. of oocytes</th>
<th>No. cleaved (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C/37°C (2.5% CO₂)</td>
<td>600</td>
<td>49 (8.1)*</td>
</tr>
<tr>
<td>37°C/39°C (2.5% CO₂)</td>
<td>550</td>
<td>152 (27.6)b</td>
</tr>
<tr>
<td>39°C/39°C (2.5% CO₂)</td>
<td>600</td>
<td>148 (24.7)b</td>
</tr>
<tr>
<td>39°C/39°C (5% CO₂)</td>
<td>610</td>
<td>480 (78.7)c</td>
</tr>
</tbody>
</table>

*Culture condition means incubation temperatures and CO₂ levels in each treatment groups: maturation temperature/fertilization temperature (CO₂ level).

*Values with different superscripts within columns are different (P < .05).
(27.6%) was significantly increased when oocytes were matured at 37°C, but fertilized at 39°C in 2.5% CO₂ (37°C/39°C and 2.5% CO₂). When oocytes were matured at 39°C and fertilized at 39°C in 2.5% CO₂ in air (39°C/39°C and 2.5% CO₂) the cleavage rate was 24.7%, which was significantly greater than 37°C/37°C and 2.5% CO₂ (P<.05), but not different from 37°C/39°C and 2.5% CO₂ (P>.05). The greatest cleavage rate (78.7%) was obtained when oocytes were matured at 39°C, fertilized at 39°C and the CO₂ level was raised to 5% (39°C/39°C, 5% CO₂).

Discussion

From previous observations made in our laboratory, it was suggested that ovary holding temperature is a possible source of variability in the success of in vitro fertilization of bovine oocytes. To test if ovary holding temperature during transportation of slaughterhouse ovaries to the embryo laboratory was detrimental, a comparison of three holding temperatures (25°C, 18°C and 0°C) was performed. The result showed a significant effect of ovary holding temperatures on oocyte maturation, fertilization and subsequent development. Temperatures below 25°C (18°C and 0°C) decreased oocyte maturation and embryo development rates, and this detrimental effect was greater at 0°C than at 18°C.

These results are in agreement with those of Yang et al. (1990), who reported the detrimental effect of holding ovaries at 4°C on cleavage and blastocyst formation compared with those transported at 25°C. However, in that experiment, they did not test any temperatures between 25°C and 4°C that could easily occur during ovary
transportation, if the temperature was not well controlled. Our results showed that oocyte maturation and embryo development were also impaired when ovaries were held at 18°C for 3 h compared with 25°C. This finding suggests that bovine oocytes are more sensitive to low temperature than mouse oocytes. Smith and Tenney (1978) have reported that the in vitro maturation of mouse oocytes was not affected by cooling murine ovaries on ice as long as 4 h before maturation incubation. The different sensitivity between these two species may be due to the difference in plasma membrane structure, organelle structure and/or composition of the cytoplasm (Pickering et al., 1990).

One of the detrimental effects of cooling oocytes that has been reported recently is disruption and disassembly of microtubules of the second meiotic spindle. Park and Ruffing (1992) have demonstrated that more than 50% of bovine IVM oocytes had reduced or absent meiotic spindles when exposed to 25°C for 1 min. Pickering et al. (1990) also reported that cooling human oocytes to room temperature for 10 min caused spindle disorganization in 50% of the oocytes examined, and less than one half of those oocytes with disorganized spindles were able to reassemble the spindle to the normal appearance following 4 h of recovery at 37°C. In contrast, our results showed no visible detrimental effect on oocyte maturation, fertilization and subsequent development when ovaries were held at 25°C for 3 h. The possible explanation may be that the cooling damage to the oocyte is developmental stage dependent. In the present study, ovaries containing germinal vesicle (GV) stage oocytes were cooled rather than metaphase-II (M-II) oocytes that were used in the study by Pickering et al. (1990) and Park and Ruffing (1992). GV-stage bovine oocytes may be able to tolerate room temperature
without being impaired for later development. M-II oocytes, however, are likely to be more sensitive to low temperature because of the presence of the meiotic spindle in which the microtubules are easily disturbed by cooling. If this is the case, the survival rates after cooling or freezing of GV-stage bovine oocytes should be better than that of M-II stage oocytes.

Another possible cause of cooling damage to the oocyte is premature cortical granule reaction, which causes reduced in vitro fertilization rates in mouse (Johnson et al., 1988). This may be true in present study since only 10% of the oocytes from ovaries held at 0°C were considered to be fertilized. This was less likely to occur in the 18°C treatment group because in vitro fertilization across matured oocytes appeared to be normal. Therefore, the reduced oocyte maturation and embryo development rates when ovaries were held at 18°C may due to some unknown factor(s), such as the changes in oocyte cytoplasm and/or membrane structure and function (Quinn, 1989).

The second experiment evaluated the effect of different combinations of incubation temperatures (37°C vs. 39°C) and CO₂ levels (2.5% vs. 5%) on in vitro fertilization of in vitro matured oocytes. A large number of oocytes were used in this experiment, and some interesting findings were generated. First, the results showed that the incubation temperature during fertilization had a significant effect on cleavage rate when the same maturation temperature and the same CO₂ level were used. The fertilization rates were 8.1% for maturation/fertilization at 37°C/37°C and 2.5%CO₂ and 27.6% for 37°C/39°C and 2.5%CO₂ (P < .0001). Secondly, when oocytes were matured at 37°C but fertilized at 39°C under 2.5%CO₂, the cleavage rate (27.6%) was as good as that of oocytes
matured at 39°C and fertilized at 39°C under 2.5% CO₂ (24.7%) (P > .05), which suggested that 37°C may be a suitable incubation temperature for bovine oocyte in vitro maturation. This finding is in partial agreement with those of Lenz et al. (1983), who reported that 39°C was significantly better than 37°C for both in vitro maturation and in vitro fertilization of bovine oocytes. Although the recommended incubation temperatures for bovine oocyte in vitro maturation and fertilization, at present, are both 39°C, which is similar to the core body temperature of the cow, the temperature of the antral follicles of female rabbit has been reported to be ~3°C lower than the internal core body temperature of the doe. Similar temperatures were found from both large and small follicles (Grinsted et al., 1980).

In vitro studies have also given evidence of a beneficial effect of 37°C on oocyte maturation in cattle compared with 39°C. Katska and Smorag (1985) reported no significant differences were found for the percentages of oocytes reaching metaphase-II after 20 h of culture at 37, 38 and 39°C. However, the results of the fluorescein diacetate (FDA) viability test showed that the fluorescein accumulation in the oocytes decreased proportionally to increased culture temperature, which indicates that the oocyte viability was decreased as culture temperature increased. Thus, the relatively low temperature in the follicles of the female (the site of oocyte maturation in vivo in most mammalian species), as well as in vitro maturation incubation may have a positive effect on oogenesis that is similar to the effect of lower temperature on spermatogenesis in the male testes (Fisch et al., 1990).
In contrast, fertilization apparently requires a higher temperature than oocyte maturation. The elevated temperature during *in vitro* fertilization seems to be able to stimulate the metabolic processes of both male and female gametes. It has been reported that the sperm capacitation and acrosome reaction are temperature dependent (Mahi and Yanagimachi, 1973). The percentage of bovine sperm cells undergoing acrosome reaction increased as the incubation temperatures were increased (Lenz et al., 1983). The sperm-oocyte fusion and post-fusion events (Hirao and Yanagimachi, 1978), as well as, the cleavage and metabolic rates of preimplantation embryos (Lavy et al., 1988; Ryan et al., 1992) have been shown to be temperature dependent. It is also well known in humans that the basal body temperature raises .5 to .8°C at the time of ovulation, which indicates that fertilization and subsequent embryo development occurs at a higher relative temperature *in vivo*. This stimulatory effect of elevated temperature on *in vitro* fertilization and subsequent embryo development, however, has been found only within a narrow temperature range. Further elevation of incubation temperature caused decreased sperm viability, percent oocyte maturation, fertilization (Lenz et al., 1983) and an increased embryo degeneration rate (Lavy et al., 1988; Ryan et al., 1992).

Oviductal fluid is rich in bicarbonate and has been shown beneficial for *in vitro* fertilization (Brackett and Mastroianni, 1974; Maas et al., 1977). Bicarbonate-buffered culture medium with 5% CO$_2$ in air (First and Parrish, 1987) or 5% CO$_2$, 5% O$_2$ and 90% N$_2$ (Younis et al., 1989) as the gas phase are commonly used for bovine *in vitro* maturation and fertilization. Since bicarbonate ion and CO$_2$ can not be separated in
current culture conditions, changes in the CO$_2$ level in gas phase will change the concentration of bicarbonate ion as well as pH of the culture medium.

Recently, contradictory findings have been reported on the effects of the CO$_2$ level on embryo development in vitro. McKierana and Bavister (1990) reported that significantly more 2-cell hamster embryos developed to the blastocyst stage with increased CO$_2$ concentration from 5 to 7.5% or 10%. However, Wang et al. (1992) reported that a significantly greater blastocyst yield was obtained under 5% CO$_2$ than 10% CO$_2$ when culturing 2- to 8-cell IVF-derived bovine embryos. In the present study, we evaluated the effects of bicarbonate and CO$_2$ on bovine in vitro fertilization by comparing maturation and fertilization incubation under 5% CO$_2$ with 2.5% CO$_2$ in air. More oocytes cleaved under 5% CO$_2$ than 2.5% CO$_2$ (78.7% vs. 24.7%; P < .0001) after in vitro maturation and fertilization at 39°C. This finding further verifies that the bicarbonate ion plays an important role during in vitro fertilization of bovine oocytes. The pH of the medium was also measured under each gas phase and a .2 pH difference (pH 7.1 under 5% CO$_2$ vs. pH 7.3 under 2.5% CO$_2$) was found. Since media at pH 7.2 to 7.4 are routinely used in mammalian embryo culture (Brinster, 1965), this stimulatory effect of CO$_2$ was unlikely due to a change in pH of the medium.

One possible explanation for this phenomena is that the higher level of CO$_2$ in the gas phase during in vitro fertilization may accelerate sperm capacitation and/or acrosome reaction. Boatman and Robbins (1991b) showed that bicarbonate and CO$_2$ affect the fertilizing ability of the hamster sperm primarily by stimulating both motility and an earlier stage of capacitation of hamster sperm cells. When bicarbonate and CO$_2$ were
continuously present, both progressive and hyperactivated motility (an indication of capacitation) were stimulated in a dose-dependent manner. It was also found that hyperactivation and zona penetration of the sperm were highly correlated. Capacitation is a series of changes in sperm cells that normally occur in the female reproductive tract, and only capacitated sperm are thought to be able to penetrate zona-intact oocytes (Chang, 1951; Austin, 1952).

Another possibility is that bicarbonate ion concentration and CO₂ levels may affect oocyte in vitro maturation. Eng et al. (1986) reported that bicarbonate ion was required for normal polar body formation. They used bicarbonate buffered medium (TCM-199E; Earle’s salts) for pig oocyte maturation and noted twice the percentage of polar body formation as the same medium based on a phosphate buffer system (TCM-199H; Hank’s salts). When the Hank’s salt based medium was supplemented with a bicarbonate buffer system, polar body formation was restored to the level in Earle’s salt based medium. In the present study, oocyte maturation status was not evaluated. However, the low level of CO₂ (2.5%) may have impaired oocyte maturation and, therefore, decreased the cleavage rate.

The low cleavage rate obtained by decreasing CO₂ level in this study may also reflect an interruption of metabolic events causing the failure of completion of fertilization and first cell division. Fixation of bicarbonate/CO₂ has been found in the preimplantation embryos of mouse (Biggers et al., 1967; Wales et al., 1969), rabbit (Kane, 1975; Quinn and Wales, 1974), and monkey (Kuehl and Dukelow, 1979). Quinn and Wales (1971, 1973) suggested that CO₂ fixation by embryos may related to the
normal enzyme activities that are required during embryogenesis. They observed a reduced incorporation of low molecular weight precursors into macromolecules when embryos were cultured in phosphate-buffered medium. Thus, a low level of CO₂ concentration in the gas phase may be necessary to ensure that there are enough bicarbonate/CO₂ can be incorporated into oocytes to complete the process of fertilization and normal cell division.

In summary, the present study has demonstrated that bovine oocytes are sensitive to low temperature. Cooling the ovary to 18°C significantly decreased oocyte maturation and subsequent embryo development rates. Furthermore, when ovaries were maintained on ice (0°C) for 3 h prior to processing, no cleavage occurred after in vitro insemination. It appears that a holding temperature at 25°C is more suitable for bovine ovary transportation than either lower or higher temperatures. Yang et al. (1990) and Sekin et al. (1992) have also reported detrimental effects of higher ovary holding temperatures (37°C and 39°C) on IVM/IVF of bovine oocytes. Our results also showed that incubation temperature and CO₂ level are very important factors on in vitro maturation and fertilization of bovine oocyte. Best cleavage rates resulted when oocytes were both matured and fertilized at 39°C in 5% CO₂. In contrast, maturation at 37°C did not decrease fertilization rate. Further study is necessary to evaluate the developmental potential of those IVF-derived bovine embryos when oocytes are matured at 37°C, fertilized and cultured for in vitro development at 39°C, since this approach may more closely mimic the physiological body conditions found in the cow.
This study clearly showed that the small changes in environmental conditions can have a big impact on oocyte *in vitro* maturation, fertilization and subsequent embryo cleavage rates. Therefore, proper handling of ovaries and control of culture conditions are essential in all embryo laboratories. In our laboratory, by employing the optimal ovary holding temperature (25°C) and *in vitro* culture conditions (39°C, 5% CO₂), 65 to 75% cleavage rates and 45 to 50% morulae were consistently produced with large numbers of oocytes (Zhang et al., 1992b; Zhang et al., 1995). Additional studies on the basic physiological conditions, as well as metabolic requirements during fertilization are needed to generate a better understanding of early embryo development. This will allow us to develop a more efficient *in vitro* system to produce bovine embryos for research and commercial animal production.
CHAPTER 5

CUMULUS CELL FUNCTION DURING BOVINE OOCYTE MATURATION, FERTILIZATION AND EMBRYO DEVELOPMENT IN VITRO

Introduction

The mammalian oocyte has a close relationship to the somatic cells during its growth and developmental phase. The cumulus-oocyte complexes (COC) are maintained by delicate cell-to-cell connections among the cumulus cells and with the oocytes (Moor et al., 1980; Eppig, 1982). The corona radiata cells form numerous intracellular processes penetrating through the zona pellucida and maintaining their communication with the oocyte via gap junctions (Anderson and Albertini, 1976; Hyttel, 1987; de Loos et al., 1991; Allworth and Albertini, 1993). This intercellular communication allows metabolite transfer (Gilula et al., 1987) and maturation (see review by Buccione et al., 1990). The breakdown of the cumulus-oocyte gap junction is correlated with oocyte meiotic resumption (Larsen et al., 1986). Ultrastructural studies demonstrated that complete disconnection between the cumulus cells and the oocyte occurs prior to oocyte maturation in vivo (Larsen et al., 1987) or in vitro (Hyttel, 1987; Suzuki et al., 1994). The exact role of the gap junctions on meiotic resumption and oocyte maturation, however, is not clearly understood (Wert and Larsen, 1989).

Numerous reports on oocyte in vitro maturation and fertilization (IVM/IVF) have suggested that oocyte appears to acquire developmental competence only when the cumulus cells are in direct contact with the oocyte (Leibfried-Rutledge et al., 1989). Significantly more cumulus-enclosed oocytes were fertilized and then developed to the
blastocyst stage \textit{in vitro} when compared with cumulus-free oocytes (Shioya et al., 1988; Mochizuki et al., 1991). A high proportion of abnormal fertilization was found when cumulus-free oocytes were used for \textit{in vitro} fertilization (Vanderhyden and Armstrong, 1989). However, all these studies involved grouping oocytes into various categories based on morphological characteristics. Some of those cumulus-free oocytes may have come from atretic follicles and were possibly degenerating at the time of collection (de Loos et al., 1991). Thus, these studies on the function of cumulus cells were likely confounded with different initial quality of the oocytes. The purpose of this study was to investigate the effect of cumulus cells and the cumulus-oocyte transzonal connections on \textit{in vitro} maturation, fertilization and subsequent embryo development in cattle. Only those oocytes with compact layers of cumulus cells and homogeneous cytoplasm were used, and the cumulus cells were removed at different developmental stages based on the experimental design for the study. These oocytes have routinely resulted in 90 to 95% maturation, 80 to 85% fertilization and 30 to 40% blastocyst development with standard procedures in our laboratories (Zhang et al., 1992b; Yang et al., 1993).

Materials and Methods

Experimental design

Experiment 5.1. Cumulus cell removal and co-culture on bovine IVM/IVF/IVC

This experiment was conducted in a 4 x 2 factorial arrangement. The effects of cumulus cell removal (at four different times) and culture condition (with or without co-culture) were investigated. Cumulus cells were removed before maturation (IVM), before insemination (IVF), 7 h post-IVF and 48 h post-IVF. The fourth treatment served
as a control; as cumulus cells were normally removed at this stage (48 h) in our routine IVF protocols (Zhang et al., 1992b; Yang et al., 1993). Denuded oocytes or embryos were then either cultured in medium alone or co-cultured with detached cumulus cells to test if the direct connection between cumulus cells and the oocyte is necessary at specific developmental stages (Table 6).

Experiment 5.2. Complete and partial cumulus cell removal on bovine IVM/IVF/IVC

This experiment was further extended to include cumulus cell removal at 7, 20, or 48 h post-IVF. Cumulus cells were either removed completely as in Experiment 1 or incompletely, with only a few cells (8 to 12 cells) remaining attached to the zona pellucida. These oocytes were either cultured in medium alone or co-cultured with cumulus cells as described in Experiment 1. The experimental outline is shown in Table 7.

Experimental oocytes

Ovaries were collected immediately after slaughter from cows or heifers at a local abattoir. Excised ovaries were placed in physiological saline (.9% NaCl) or Dulbecco's phosphate-buffered saline (DPBS) and maintained at 23 to 28°C during transport to the embryo laboratory. In the laboratory, ovaries were rinsed three times in DPBS containing antibiotics (100 IU penicillin, 100 μg streptomycin/ml) and dried with a sterile paper towel. Oocytes were aspirated from small antral follicles (2 to 8 mm in diameter) using an 18-gauge needle attached to a 6 ml sterile, plastic syringe. Oocytes with intact cumulus and homogeneous cytoplasm were selected and randomly assigned to various treatments in these experiments.
Table 6. Experimental design for Experiment 5.1

<table>
<thead>
<tr>
<th>Cumulus cell removal</th>
<th>Co-culture</th>
<th>No. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before IVM</td>
<td>Yes</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>200</td>
</tr>
<tr>
<td>Before IVF</td>
<td>Yes</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>200</td>
</tr>
<tr>
<td>7 h after IVF</td>
<td>Yes</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>200</td>
</tr>
<tr>
<td>48 h after IVF</td>
<td>Yes</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>200</td>
</tr>
</tbody>
</table>
Table 7. Experimental design for Experiment 5.2

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cumulus cell removal</th>
<th>Co-culture</th>
<th>No. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete cell removal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 h after IVF</td>
<td>Yes</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>20 h after IVF</td>
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<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>100</td>
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</tr>
<tr>
<td>48 h after IVF</td>
<td>Yes</td>
<td>100</td>
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<tr>
<td>Partial cell removal</td>
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<td></td>
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<td>20 h after IVF</td>
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<td></td>
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<td>100</td>
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</tr>
</tbody>
</table>
Cumulus cell removal

Cumulus cells were removed from oocytes or early embryos by placing them in 2.9% sodium citrate solution for 2 min followed by manual shaking or vortexing. The detached cumulus cells were recovered to prepare monolayers. Only those oocytes or early embryos without any attached cumulus cells were used in these experiments. Denuded oocytes or embryos were then processed for IVM/IVF and cultured in medium alone or in co-culture with the detached cumulus cells (1 × 10^6 cells/ml) as indicated in Experiments 5.1 and 5.2. The co-culture groups of oocytes or embryos were continuously cultured on the cumulus cell monolayers except during in vitro fertilization.

In vitro maturation and fertilization

Standard successful procedures used routinely in our laboratories (Zhang et al., 1992; Yang et al., 1993) were employed for experiments. Oocytes were placed in 500 μl medium in four-well tissue culture plates (Nunclon, 25 oocytes/well, Experiment 1) or 100 μl droplets (15 oocytes/droplet, covered with Dow Corning Medical fluid, Experiment 2). Tissue Culture Medium-199 (TCM-199) supplemented with 5% fetal bovine serum (FBS) was used throughout the study for maturation and embryo culture. The oocytes were matured for 22 h and the culture environment was 5% CO₂ in humidified air at 39°C.

At 1 to 2 h prior to insemination, two straws of frozen semen from a fertile dairy bull were thawed in a 25°C water bath for 1 min. The sperm cells were washed twice in B-O medium (Brackett and Oliphant, 1975) supplemented with 10 mM caffeine by centrifugation (350 x g, 6 min). The sperm cell concentration was adjusted to 3 × 10^6
motile sperm/ml for the experiments. Sperm cells were then exposed to .1 μM of Ca++ ionophore A23187 for 1 min to aid in capacitation. Approximately 50 μl of the treated sperm cells were quickly added to each 50 μl fertilization droplet containing oocytes. The fertilization medium was B-O medium containing 20 mg/ml bovine serum albumin (BSA). The final sperm concentration was 1.5 x 10⁶ motile sperm/ml. Oocytes and sperm cells were co-incubated at 39°C with 5% CO₂ in humidified air for 6 h.

Embryo culture

After 6 h of co-incubation, the inseminated oocytes were transferred to TCM-199 with 5% FBS in four-well tissue culture plates or microdroplets and cultured for an additional 42 h under the same condition to assess cleavage. Cleaved embryos were continuously cultured in the same conditions for 10 to 14 days. The culture medium was changed every 2 days and embryo development was recorded. All uncleaved ova were fixed and stained with 2% orcein to examine the arrested stages.

Statistical analysis

The criteria for evaluation included percentages of oocyte maturation, fertilization, and embryo development to cleavage, morula, blastocyst and hatched blastocyst stages. The results were analyzed with SAS categorical data modeling (CATMOD) procedure (SAS Institute, Gary, NC) and the logit model was used to fit the observed data. A P value of <.05 was regarded as statistically significant and the best-fitted model was used for comparisons. The differences among individual treatments were assayed by developing 95% confidence intervals.
Results

Experiment 1

In this experiment, the effect of cumulus cells and their cellular connection with the oocyte on IVM/IVF was tested by removing the cumulus cells from the oocytes at various stages and then culturing the oocytes or embryos with or without the detached cumulus cells.

The results of oocyte \textit{in vitro} maturation, fertilization and subsequent embryo development rates are shown in Table 8. Overall, cumulus cell removal significantly affected oocyte fertilization and embryo development ($P < .0001$). There was no problem in removing the cumulus cells from the oocytes either before or after maturation with our procedure. Removing cumulus cells with enzyme such as hyaluronidase treatment was not successful for immature oocytes, thus, we abandoned this approach in the experiment. When cumulus cells were removed before maturation culture, only 4 to 26\% of the oocytes developed to the metaphase II (M-II) stage, and even poorer fertilization and development resulted when compared with other treatment groups ($P < .05$). No interaction ($P > .05$) between cumulus cell removal and developmental stages was detected indicating that in this experiment, no matter at what stage that the cumulus cells were removed, it affected negatively on fertilization and subsequent development when compared with the control group ($P < .05$).

The culture condition also significantly affected embryo development ($P < .0001$). Higher development rates to morula and blastocyst stages were obtained when denuded oocytes or embryos were co-cultured with detached cumulus cells versus without cells.
Table 8. Effects of cumulus cell removal at various times and co-culture on oocyte maturation, fertilization and embryo development*

<table>
<thead>
<tr>
<th>Cumulus cells removed</th>
<th>Co-culture</th>
<th>No. of oocytes</th>
<th>% of oocytes developed to*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td></td>
<td>MATR</td>
</tr>
<tr>
<td>Before IVM</td>
<td>Yes</td>
<td>219</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>216</td>
<td>4</td>
</tr>
<tr>
<td>Before IVF</td>
<td>Yes</td>
<td>192</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>196</td>
<td>92</td>
</tr>
<tr>
<td>7 h after IVF</td>
<td>Yes</td>
<td>177</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>188</td>
<td>93</td>
</tr>
<tr>
<td>48 h after IVF</td>
<td>Yes</td>
<td>202</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>162</td>
<td>93</td>
</tr>
</tbody>
</table>

♦Major results of analysis: effect of cumulus cell removal (P < .0001); co-culture effect (P < .0001); interaction of cumulus cell removal and co-culture (P < .01).

*Abbreviations: MATR=matured, FERT=fertilized, CLEAV=cleaved, MORL=morula, BLST=blastocyst, HBLST=hatched blastocyst.
(P < .05, Table 8). The interaction between culture conditions and developmental stages was significant (P < .01). The beneficial effect of cumulus cell co-culture was more prominent at later embryonic stages than at early stages. The interaction between cumulus cell removal and culture condition was also significant (P < .01). The interaction was partially introduced by the experimental procedure. The earlier the cumulus cells were removed from the oocytes, the stronger the co-culture effect.

To more clearly show the effect of cumulus removal on each stage of development, the statistical analyses of the main effects are presented in Table 9 and 10. The percentages of development showed in these tables were based on their previous developmental stages. This helped eliminate the interference of early treatment effect on later developmental stages. Table 9 shows the result of removing cumulus cells at different stages in the presence of co-culture with cumulus cells. Removing cumulus cells from oocytes before maturation significantly (P < .05) reduced the rates of oocyte maturation, fertilization and embryo developmental potential in each progressive stage, except for the development from morula to blastocyst stages (P < .05). When cumulus cells were removed after maturation, but before IVF or 7 h after IVF, fewer oocytes were fertilized (P < .05), but the subsequent development rates of the fertilized ova were not affected (P < .05). More oocytes were fertilized when cumulus cells were removed at 7 h after IVF compared with those with cumulus cells removed before IVF (P < .05). The effects of cumulus cell removal in the absence of co-culture are shown in Table 10. The overall developmental rates were lower compared with the corresponding treatments of the co-culture groups (P < .05). Negative impact on development by cumulus cell
Table 9. Effect of cumulus cell removal at various times on the development of IVF-derived bovine embryos in the presence of co-culture $^a$

<table>
<thead>
<tr>
<th>Cumulus cells removed</th>
<th>No. of oocytes</th>
<th>% of oocytes developed to $^b$</th>
<th>MATR</th>
<th>FERT</th>
<th>CLEAV</th>
<th>MORL</th>
<th>BLST</th>
<th>HBLST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before IVM</td>
<td>219</td>
<td></td>
<td>26$^e$</td>
<td>35$^e$</td>
<td>50$^e$</td>
<td>20$^e$</td>
<td>100$^e$</td>
<td>0$^e$</td>
</tr>
<tr>
<td>Before IVF</td>
<td>192</td>
<td></td>
<td>93$^d$</td>
<td>62$^d$</td>
<td>81$^{de}$</td>
<td>40$^d$</td>
<td>31$^e$</td>
<td>55$^d$</td>
</tr>
<tr>
<td>7 h after IVF</td>
<td>177</td>
<td></td>
<td>93$^d$</td>
<td>76$^e$</td>
<td>76$^d$</td>
<td>50$^d$</td>
<td>46$^e$</td>
<td>50$^d$</td>
</tr>
<tr>
<td>48 h after IVF</td>
<td>202</td>
<td></td>
<td>96$^d$</td>
<td>95$^f$</td>
<td>85$^e$</td>
<td>61$^d$</td>
<td>40$^e$</td>
<td>64$^d$</td>
</tr>
</tbody>
</table>

$^a$Data presented were derived from Table 8. Denuded ova were co-cultured with detached cumulus cells.

$^b$The percentage of development is based on the previous stages.

$^{c,d,e,f}$Values with different superscripts within columns are different ($P < .05$).
Table 10. Effect of cumulus cell removal at various times on the development of IVF-derived bovine embryos in the absence of co-culture\textsuperscript{a}

<table>
<thead>
<tr>
<th>Cumulus cells removed</th>
<th>No. of oocytes</th>
<th>% of oocytes developed to\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before IVM</td>
<td>216</td>
<td>MATR 4\textsuperscript{a} FERT 0\textsuperscript{a} CLEAV 0\textsuperscript{a} MORL 0\textsuperscript{a} BLST 0\textsuperscript{a} HBLST 0\textsuperscript{a}</td>
</tr>
<tr>
<td>Before IVF</td>
<td>196</td>
<td>92\textsuperscript{d} 66\textsuperscript{d} 66\textsuperscript{d} 29\textsuperscript{d} 0\textsuperscript{a} 0\textsuperscript{a}</td>
</tr>
<tr>
<td>7 h after IVF</td>
<td>188</td>
<td>93\textsuperscript{d} 77\textsuperscript{e} 74\textsuperscript{e} 40\textsuperscript{d} 8\textsuperscript{de} 0\textsuperscript{e}</td>
</tr>
<tr>
<td>48 h after IVF</td>
<td>162</td>
<td>93\textsuperscript{d} 97\textsuperscript{f} 82\textsuperscript{e} 57\textsuperscript{e} 22\textsuperscript{e} 40\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data presented were derived from Table 8. Denuded ova were cultured in medium alone.

\textsuperscript{b}The percentage of development is based on the previous stages.

\textsuperscript{c,d,e,f}Values with different superscripts within columns are significantly different (P < .05).
removal without co-culture was detected at practically all subsequent stages of development (see Tables 9 and 10).

**Experiment 2**

This experiment was an extension of Experiment 5.1 by testing the effect of cumulus cell removal (complete vs. partial) at 7, 20 and 48 h after IVF (Table 11). When cumulus cells were partially removed but with only a few cumulus cell remaining attached to the zona pellucida, no differences ($P > .05$) were noted among the treatment groups at all stages examined, except at the hatched blastocyst stage where co-culture enhanced the rate of embryo development ($P < .05$) and cell count (not shown). When cumulus cells were completely removed (Experiment 5.2), significantly more embryos developed to morula stage or beyond for the 20- and 48-h treatment groups compared with the 7-h group. No difference ($P > .05$) was detected between the 20-h vs. 48-h treatment groups in all stages examined, particularly when cumulus cell co-culture was used during incubation. Cumulus cell co-culture did not affect fertilization or cleavage development of embryos ($P > .05$), but did significantly improve embryo development to morula stage or beyond for all three different cumulus cell removal time treatment groups examined ($P < .05$). No interaction between stage of cumulus cell removal and co-culture was found in this study ($P > .05$).

Because no difference was noted among replications within experiment and the same procedures were used for both studies, except for the extent of cumulus cell removal, data were pooled for a retrospective comparison between the extent of cumulus cell removal on subsequent embryo development. The pooled results and their
Table 11. Effect of cumulus cells on fertilization and embryo development

<table>
<thead>
<tr>
<th>Cumulus removal</th>
<th>Co-culture</th>
<th>No. of oocytes</th>
<th>% of oocytes developed to</th>
<th>FERT</th>
<th>CLEAV</th>
<th>MORL</th>
<th>BLST</th>
<th>HBLST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete removal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 h IVF</td>
<td>Yes</td>
<td>90</td>
<td>81</td>
<td>70</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>86</td>
<td>81</td>
<td>64</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>20 h IVF</td>
<td>Yes</td>
<td>93</td>
<td>88</td>
<td>73</td>
<td>30</td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>92</td>
<td>89</td>
<td>80</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>48 h IVF</td>
<td>Yes</td>
<td>93</td>
<td>88</td>
<td>76</td>
<td>30</td>
<td>19</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>83</td>
<td>84</td>
<td>77</td>
<td>25</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Partial removal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 h IVF</td>
<td>Yes</td>
<td>91</td>
<td>89</td>
<td>80</td>
<td>34</td>
<td>21</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>90</td>
<td>83</td>
<td>68</td>
<td>27</td>
<td>18</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>20 h IVF</td>
<td>Yes</td>
<td>90</td>
<td>86</td>
<td>77</td>
<td>34</td>
<td>18</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>92</td>
<td>88</td>
<td>75</td>
<td>38</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>48 h IVF</td>
<td>Yes</td>
<td>97</td>
<td>89</td>
<td>79</td>
<td>38</td>
<td>24</td>
<td>5</td>
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<td>93</td>
<td>89</td>
<td>76</td>
<td>39</td>
<td>27</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Major result of analysis: for complete removal of cumulus cells, significant differences (P < .05) were noted at morula stage or beyond by cumulus cell removal and co-culture, and for incomplete removal of cumulus cells, no difference was detected among treatment groups at all stages evaluated, except for hatched blastocyst stage where co-culture improved development (P < .05). Interactions between stage of cumulus cell removal and co-culture are not significantly different (P > .05).*
comparisons are presented in Table 12. The extent of cumulus cell removal did not affect the rate of fertilization or cleavage development (P > .05). However, partial cumulus cell removal allowed more cleaved embryos to develop to the morula or blastocyst stage (P < .05), particularly when removal was performed at 7 or 20 h after IVF when compared with complete cumulus cell removal. Partial cumulus cell removal resulted in similar rates of progressive embryo development regardless of the stages of cumulus removal (P > .05).

Discussion

Previous reports have suggested the importance of cumulus cells during bovine oocyte in vitro maturation and fertilization after studying different populations of oocytes with different layers of cumulus cell. Significantly lower rates of maturation and fertilization were obtained from the group of cumulus-free oocytes compared with cumulus-enclosed oocytes (Fukui and Sakuma, 1980; Shioya et al., 1988; Leibfried-Rutledge, et al., 1989). However, the cumulus-free oocytes used in those studies may have originated from atretic follicles and, therefore, could have already started to degenerate prior to in vitro culture. In the present study, only cumulus intact, morphologically normal-appearing oocytes were selected and used in experiments. Cumulus cells were removed at different developmental stages and then replaced in the culture medium to evaluate the effect of cumulus cells on oocyte maturation, fertilization and early embryonic development in vitro. The results demonstrated that the intact state of surrounding cumulus cells of oocytes are beneficial at least up to 7 h after IVF, but not at 20 h after IVF.
Table 12. Main effect of partial and complete cumulus cell removal

<table>
<thead>
<tr>
<th>Cumulus cell removal</th>
<th>No. of oocytes</th>
<th>Progressive % of ova developed to</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FERT</td>
<td>CLEAV</td>
<td>MORL</td>
<td>BLST</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>Extent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 h IVF</td>
<td>Complete</td>
<td>176</td>
<td>143 (81)*</td>
<td>118 (83)*</td>
<td>23 (19)*</td>
<td>2 (9)*</td>
</tr>
<tr>
<td></td>
<td>Partial</td>
<td>181</td>
<td>156 (86)*</td>
<td>134 (86)*</td>
<td>56 (42)b</td>
<td>36 (64)b</td>
</tr>
<tr>
<td>20 h IVF</td>
<td>Complete</td>
<td>185</td>
<td>164 (89)*</td>
<td>142 (87)*</td>
<td>40 (28)c</td>
<td>16 (40)c</td>
</tr>
<tr>
<td></td>
<td>Partial</td>
<td>182</td>
<td>158 (87)*</td>
<td>138 (87)*</td>
<td>66 (48)b</td>
<td>38 (58)b</td>
</tr>
<tr>
<td>48 h IVF</td>
<td>Complete</td>
<td>176</td>
<td>152 (86)*</td>
<td>135 (89)*</td>
<td>49 (36)bc</td>
<td>24 (49)bc</td>
</tr>
<tr>
<td></td>
<td>Partial</td>
<td>186</td>
<td>169 (91)*</td>
<td>148 (88)*</td>
<td>73 (49)b</td>
<td>48 (66)b</td>
</tr>
</tbody>
</table>

Values with different superscripts within columns are significantly different (P < .05).
Cumulus cell removal before maturation appears to be detrimental to bovine oocytes. Only 4% of the denuded oocytes developed to M-II stage after 22 h of culture in medium alone, and most of the oocytes degenerated during this culture period. When denuded oocytes were co-cultured with detached cumulus cells, the maturation rate was significantly improved to 26%. However, when intact cumulus-oocyte complexes were matured in the same condition, on the average, 93% of oocytes developed to M-II stage, similar to the results of previous studies (Zhang et al., 1992b; Yang et al., 1993). Because the evaluation of maturation was conducted until after fertilization, sufficient time may have elapsed to allow oocytes to degenerate and therefore appear not to have reached metaphase II. Whereas it is possible to underestimate the "true" maturation rates, the fact that all groups were evaluated at the same time makes the comparison valid and meaningful. This result thus indicates that the intact stage of the cumulus-oocyte complex is essential and important during oocyte maturation in cattle.

Ultrastructural analysis of bovine cumulus-oocyte complexes also demonstrate that a complete breakdown of the cytoplasmic processes occurs prior to oocyte maturation at 13 to 20 h of in vitro maturation (Hyttel, 1987; Laurincik et al., 1992; Suzuki et al., 1994). In contrast, a more recent study (Allworth and Albertini, 1993) with laser scanning confocal and fluorescence microscopy demonstrated that complete breakdown of cytoplasmic processes did not occur even at 24 h of maturation. However, the transition between actin-rich and microtubule-rich processes was evident during different stages of maturation. It has been hypothesized that follicular cells produce meiosis-arresting factors and that the breakdown of these junctional complexes causes meiotic
resumption. The findings of this study demonstrated that complete cumulus cell removal blocked maturation and co-culture of oocytes with detached cumulus cells partially restored maturation suggesting other possible factors may be involved in oocyte maturation.

Research in cattle and other species also showed a beneficial effect of cumulus cells on *in vitro* maturation and fertilization (Kennedy and Donahue, 1969; Cross and Brinster, 1970; Vanderhyden and Armstrong, 1989; Chian and Niwa, 1994). Although some denuded oocytes can complete the meiotic resumption, fertilization and development to normal cleaved embryos is significantly reduced as shown in the present study and by others in previous reports (Staigmiller and Moor, 1984; Sirard et al., 1988). It has also been noted that a higher proportion of fertilized oocytes that matured without cumulus cells showed evidence of abnormal fertilization (Vanderhyden and Armstrong, 1989; Chian and Niwa, 1994).

Our results showed a significant decrease in fertilization rate when cumulus cells were removed prior to insemination. This negative effect was also noted when cumulus cells were removed 7 h after insemination in Experiment 5.1, but was not evident in Experiment 5.2. Interestingly, even though cumulus cells removed at this time decreased the fertilization rate compared with those in the control groups, subsequent development of the fertilized ova seemed not to be affected. Similar rates of embryo development to blastocyst and hatched blastocyst stages were obtained when compared with the control groups, particularly in the co-culture groups (*P* > .05, Table 9). This indicates that the overall low development rates in these treatment groups (Table 8) were primarily due to
the reduced rates of fertilization. However, the beneficial effect of cumulus cells on fertilization and its mechanisms are still unclear. Possible explanations include: 1) cumulus cells are able to facilitate sperm cell penetration and fertilization (Brackett et al., 1989; Vanderhyden and Armstrong, 1989; Fukui, 1990; Mochizuki, 1991; Younis and Brackett, 1991); 2) cumulus cells are able to induce sperm capacitation and acrosome reaction in cattle (Fukui, 1990; Boatman and Robbins, 1991a; Takahashi and First, 1993); and 3) cumulus cells facilitate sperm penetration and fertilization by preventing zona hardening (Downs et al., 1986; Mattioli et al., 1988).

Despite all the supporting evidence on the beneficial effect of cumulus cells, two recent studies require some attention. Hawk et al. (1992) reported that partially detaching cumulus cells from bovine oocytes either before or after maturation significantly increased fertilization rate and the proportions of oocytes developing to expanded blastocysts. Dominko and First (1991) suggested that maturation of cumulus-removed oocytes may result in normal fertilization and embryo development when cultured on a cumulus cell monolayer. One possible explanation for these findings is incomplete removal of cumulus cells from oocytes in these experiments. These remaining cumulus-oocyte connections may have been sufficient to provide the communication between the oocyte and cumulus cells, as suggested by the present study (Experiment 5.2). To what extent the cumulus cells should be removed remains a question for further investigation.

Presence of cumulus cells in the culture system significantly improved embryo development to morula, blastocyst and hatched blastocyst stages in this study. This supports previous reports on the beneficial effects on mammalian embryos of co-culture
or conditioned medium (Eyestone and First, 1989; Kane et al., 1992; see review by Thibodeaux and Godke, 1992). However, Bavister et al. (1992) have reported similar rates of morula/blastocyst development in TCM-199 alone and conditioned medium. Our present study suggests that a few remaining cumulus cells on the zona may mask the difference and support embryo development beyond the in vitro culture block. In our laboratories, cumulus cells are routinely used as co-culture cells for culturing bovine IVF embryos (Zhang et al., 1992b; Yang et al., 1993). Live calves have been born after transfer of frozen-thawed IVF-derived cumulus cell co-cultured embryos (Zhang et al., 1993). These experiments and those of other investigators (Goto et al., 1988; Fukuda et al., 1990) have shown that the cumulus cell co-culture system at present, remains better in supporting embryonic development over the control medium alone.

In conclusion, this study demonstrated that cumulus cells are important and necessary for oocyte maturation and acquisition of full embryonic development competence. The intact stage of the surrounding cumulus cells was demonstrated to be beneficial to the oocyte and subsequent embryo development at least up to 7 h, but not at 20 h after insemination of oocytes. The efficiency of producing IVF-derived bovine embryos was comparable to the controls when cumulus cells were completely removed \( \approx 20 \) h post-insemination. The results also showed that co-culture of denuded oocytes improved the rates of maturation and fertilization through some unknown mechanism(s). However, this improvement did not overcome the detrimental effect caused by breakdown of the direct connection. The results with embryo co-culture confirmed
previous findings of the beneficial effect of somatic cells on overcoming the 8- to 16-cell

*in vitro* "developmental block" and enhanced subsequent embryo development.
EVALUATING BOVINE OVIDUCT CELLS USED IN COMBINATION WITH BOVINE CUMULUS CELLS TO CO-CULTURE IVF-DERIVED BOVINE EMBRYOS IN VITRO

Introduction

Although the biochemical processes during co-culture and the interaction between embryo and "helper" cells are not yet fully understood, various types of somatic cells have been shown to benefit early bovine embryos development in vitro (Kuzan and Wright, 1982; Camous et al., 1984; Lu et al., 1987b; Goto et al., 1988; Scodras et al., 1991). Pregnancies and normal calves have been produced after transfer of in vitro matured/in vitro fertilized (IVM/IVF) oocytes subsequently in vitro cultured (IVC) with bovine oviduct cells (Lu et al., 1988; Xu et al., 1990; Monson et al., 1992) and bovine cumulus cells (Goto et al., 1988; Zhang et al., 1992a). However, the proportion of IVM/IVF embryos that develop to blastocyst stages in different in vitro culture systems remains variable, suggesting that the optimum culture system for IVF-derived bovine embryos has yet to be discovered.

Conventional culture systems for in vitro development of early stage bovine embryos are somewhat complicated, involving a complex culture medium (e.g. TCM-199 or Ham's F-10), serum, and somatic cells. Recently, several research groups reported using simple chemically defined, serum-free medium to support the in vitro development of early stage in vivo fertilized (Ellington et al., 1990a) or IVF-derived (Pinyopummintr and Bavister, 1991) bovine embryos. It would be beneficial to eliminate undefined
biochemical components in standard complex culture media so that the embryotrophic components produced by co-culture cells could be analyzed and the requirements for early embryo development could be identified.

The objective of this study was to evaluate the use of bovine oviduct cells, bovine cumulus cells and a combination of both of these cells to co-culture IVF-derived bovine embryos. In addition, two different culture media, modified CZB medium, a chemically defined serum-free medium containing 20 amino acids and TCM-199, a complex medium, supplemented with 5% fetal bovine serum (FBS) were also compared for supporting IVF-derived embryos in both co-culture and medium alone culture.

Materials and Methods

**IVF-derived embryos**

Cumulus-intact oocytes were harvested from 3 to 8 mm follicles from abattoir ovaries of dairy and beef females. The oocytes with compacted cumulus cell were selected and washed two times in Dulbecco's phosphate-buffered saline (DPBS) containing .1% Polyvinyl alcohol (PVA) and two times in Tissue Culture Medium 199 (TCM-199) plus 5% fetal bovine serum (FBS). Oocytes were then incubated in .5 ml of TCM-199 with 5% FBS at 39°C for 24 h. At 1 h prior to insemination, two straws of frozen semen of a fertile dairy bull were thawed in a 25°C water bath for 1 min. The sperm cells were washed with Brackett-Oliphant (B-O) medium containing 10 mM of caffeine and adjusted to a concentration of 3 x 10⁶ motile sperm cells/ml. Sperm cells were then exposed to .1 μM of Ca⁺⁺ ionophore A23187 for 1 min to aid in capacitation. Approximately 50 μl of a sperm cell suspension was then quickly added to each 50 μl
fertilization droplet containing 20 to 25 oocytes. The fertilization medium was B-O medium containing 20 mg/ml bovine serum albumin (BSA). Oocytes and sperm cells were co-incubated at 39°C in 5% CO₂ in humidified air. After 6 h of incubation, the oocytes (with their cumulus cells) were moved to TCM-199 with 5% FBS and incubated for ≈40 h until they reached the 2- to 8-cell stage. Cumulus cells were removed from these early IVM/IVF embryos by culturing them in 2.9% sodium citrate solution for 2 min followed by manual shaking. Only early stage embryos without any attached cumulus cells were used in this experiment.

Experimental design

This experiment was conducted according to a 2 x 4 factorial arrangement. The effects of two culture media: 1) CZB medium; 2) TCM-199 plus 5% FBS and four culture systems: 1) medium alone as a control; 2) bovine oviduct epithelia cell co-culture (BOEC); 3) bovine cumulus cell co-culture (BCC); and 4) BOEC + BCC co-culture were evaluated in this experiment. Two- to 8-cell stage IVF-derived bovine embryos were randomly assigned into each of eight treatment groups. The experimental outline is shown in Table 13.

Monolayer preparation and co-culture

Bovine oviducts (obtained from midcycle mature beef females at a local abattoir) were transported at 25°C in PBS with antibiotics (1000 IU penicillin and 1000 μg streptomycin/ml PBS). Oviducts were washed twice in PBS, dried with a sterile paper towel, trimmed free from surrounding connective tissue, briefly dipped in 70% ethanol, rinsed in PBS and again dried. The infundibulum was excised from each oviduct.
Table 13. Experimental design for Experiment 6

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Culture system</th>
<th>No. of 2- to 8-cell embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>CZB</td>
<td>Medium alone</td>
<td>100</td>
</tr>
<tr>
<td>CZB</td>
<td>BOEC</td>
<td>100</td>
</tr>
<tr>
<td>CZB</td>
<td>BCC</td>
<td>100</td>
</tr>
<tr>
<td>CZB</td>
<td>BOEC+BCC</td>
<td>100</td>
</tr>
<tr>
<td>TCM-199</td>
<td>Medium alone</td>
<td>100</td>
</tr>
<tr>
<td>TCM-199</td>
<td>BOEC</td>
<td>100</td>
</tr>
<tr>
<td>TCM-199</td>
<td>BCC</td>
<td>100</td>
</tr>
<tr>
<td>TCM-199</td>
<td>BOEC+BCC</td>
<td>100</td>
</tr>
</tbody>
</table>
Epithelial cells were manually expressed into a sterile test tube by gently squeezing the oviduct between the jaws of a pair of sterile forceps while pulling from the isthmus toward the infundibulum. The harvested cells were washed three times with TCM-199 plus 5% FBS by centrifugation (360 x g, 4 min). The cell pellets were resuspended in the same medium, and the cells were seeded in four-well tissue culture plate (1 x 10^6 cells/ml) to develop monolayers for BOEC co-culture. The culture plates that used for developing early stage IVF embryos (cumulus cells monolayer formed at this time) were used in BCC cell and BOEC+BCC cell co-culture treatments (Figure 3). The oviduct cell clumps was used in BOEC+BCC cell co-cultures.

Early stage embryos free of cumulus cells (n=800) were equally and randomly allotted to one of the eight treatment groups.Embryos were incubated in .5 ml of CZB medium (Appendix C) or TCM-199 (25 Mm HEPES, Earle's salts, GIBCO) plus 5% FBS either alone or with medium plus cells. The culture media were replaced with fresh medium at 48-h intervals and embryos were evaluated using an inverted microscope immediately after changing the medium. There were four replications in this experiment.

Statistical analysis

Chi-square (χ^2) analysis was used to test the difference in the proportion of oocytes developed to morula, blastocyst, expanded blastocyst and hatched blastocyst stages. A P-value of < .05 was regarded as statistically significant in this experiment.

Results

In this experiment, the effect of two culture media (CZB medium and TCM-199) and two type of "helper" cells (BOEC and BCC) on supporting IVF-derived 2- to 8- cell
Figure 3. Somatic cell monolayers used for co-culture of IVF-derived bovine embryos. Bovine cumulus cell (BCC) monolayer (a). Bovine oviduct epithelial cell (BOEC) monolayer (b). Magnification: X600.
stage bovine embryos was tested. Within each culture medium, embryos were either cultured in medium alone or co-cultured with BOEC, BCC and BOEC + BCC. The results of embryo development rates at morula, blastocyst, expanded blastocyst and hatched blastocyst stages are shown in Table 14.

When CZB medium was used as a culture medium, more 2- to 8-cell stage embryos developed to blastocysts and expanded blastocysts after co-culturing with BOEC, BCC and BOEC + BCC treatment groups (P < .05) than culturing in CZB medium alone. When TCM-199 with 5% FBS was used, more embryos developed to blastocysts and expanded blastocysts (P < .05) in the BCC cell and the BOEC + BCC cell co-culture treatment groups when compared with embryos in the BOEC cell co-culture or in medium alone culture. This pattern continued through to the embryo hatching stage.

To compare overall medium effect, data from both co-cultures and medium alone culture were pooled according the culture medium that was used. Result showed that more 2- to 8-cell stage embryos developed to morula stage in CZB (P < .05) than in TCM-199 with serum (74% vs. 66%). However, fewer embryos hatched in vitro in CZB medium without serum (P < .05) than TCM-199 with serum (6% vs. 14%). No significant differences were found between CZB and TCM-199 on supporting IVF-derived embryo development at blastocyst and expanded blastocyst stages (P > .05).

Discussion

The in vitro development of IVF-derived bovine embryos in co-culture with bovine oviduct epithelial cell, bovine cumulus cell and the combination of these two types of cells using both simple serum-free medium (CZB medium) and complex serum-
Table 14. Effect of cell types and culture media on \textit{in vitro} development of IVF-derived bovine embryos*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of 2- to 8-cells</th>
<th>No. of MORL (%)</th>
<th>No. of BLST (%)</th>
<th>No. of ExBLST (%)</th>
<th>No. of HBLST (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CZB (control)</td>
<td>100</td>
<td>68(^a)</td>
<td>14(^a)</td>
<td>9(^a)</td>
<td>2(^a)</td>
</tr>
<tr>
<td>CZB + BOEC</td>
<td>100</td>
<td>68(^a)</td>
<td>26(^b)</td>
<td>20(^b)</td>
<td>4(^a,b)</td>
</tr>
<tr>
<td>CZB + BCC</td>
<td>100</td>
<td>81(^b)</td>
<td>35(^b)</td>
<td>29(^b)</td>
<td>8(^a,b)</td>
</tr>
<tr>
<td>CZB + BOEC + BCC</td>
<td>100</td>
<td>78(^a,b)</td>
<td>31(^b)</td>
<td>26(^b)</td>
<td>11(^b)</td>
</tr>
<tr>
<td>TCM-199 (control)</td>
<td>100</td>
<td>68(^a,b)</td>
<td>18(^a)</td>
<td>15(^a,b)</td>
<td>6(^a)</td>
</tr>
<tr>
<td>TCM-199 + BOEC</td>
<td>100</td>
<td>55(^a)</td>
<td>16(^a)</td>
<td>9(^a)</td>
<td>7(^a)</td>
</tr>
<tr>
<td>TCM-199 + BCC</td>
<td>100</td>
<td>69(^b)</td>
<td>28(^b)</td>
<td>27(^b)</td>
<td>22(^b)</td>
</tr>
<tr>
<td>TCM-199 + BOEC + BCC</td>
<td>100</td>
<td>71(^b)</td>
<td>26(^b)</td>
<td>26(^b)</td>
<td>22(^b)</td>
</tr>
<tr>
<td>Total CZB combined</td>
<td>400</td>
<td>(74)(^a)</td>
<td>(27)(^a)</td>
<td>(21)(^a)</td>
<td>(6)(^a)</td>
</tr>
<tr>
<td>Total TCM combined</td>
<td>400</td>
<td>(66)(^b)</td>
<td>(22)(^a)</td>
<td>(19)(^a)</td>
<td>(14)(^b)</td>
</tr>
</tbody>
</table>

*MORL = morulae; BLST = blastocysts; ExBLST = expanded blastocysts; HBLST = hatched blastocysts.

\(^{a,b}X^2\) analysis was used to compare treatment difference within each column for each culture medium (P < .05).
containing medium (TCM-199 plus 5% FBS) were studied in this experiment. The results clearly showed the evidence of the beneficial effect of co-culture on embryo development. Significantly more embryos developed to morula, blastocyst and hatched blastocyst stages from all three co-culture treatment groups compared with medium alone culture when CZB medium was used. However, when TCM-199 plus 5% FBS was used for embryo culture, more embryos developed to morulae, blastocysts and hatched blastocysts in cumulus cells co-culture and oviduct cell plus cumulus cell co-culture compared with in oviduct cell co-culture and in medium alone culture. These data suggest that bovine cumulus cells with or without bovine oviduct cells would be an acceptable choice for co-culturing IVF-derived bovine embryos.

Studies on bovine in vitro fertilization have also shown the importance of cumulus/granulosa cells on oocyte in vitro maturation and fertilization (Leibfried-Rutledge et al., 1989; Younis and Brackett, 1991). Greater maturation and fertilization rates were observed with the use of cumulus-enclosed bovine oocytes than cumulus-denuded oocytes (Shioya et al., 1988; Mochizuki et al., 1991; Cox et al., 1993). Removal of cumulus cells before oocyte maturation and fertilization resulted in lower maturation and fertilization rates (see Chapter 5), whereas addition of granulosa cells to culture medium improved the fertilization rates (Mochizuki et al., 1991).

The in vitro development of IVF-derived bovine embryos were also enhanced by using cumulus cell co-culture. Goto et al. (1988) reported the establishment of pregnancies after nonsurgical transfer of IVF-derived blastocyst stage bovine embryos that were co-cultured with cumulus cells. Younis and Brackett (1991) reported that 20% of IVF-derived embryos developed to morula and blastocyst stages when co-cultured with
cumulus cells compared with only 2.8% when cultured in medium alone. The results from the present study further confirmed those previous findings, and showed the supporting effect of cumulus cell co-culture on the in vitro development of IVF-derived 2- to 8-cell stage embryos to morula and blastocyst stages in both simple serum-free medium (CZB) and complex serum-containing medium (TCM-199).

Oviduct epithelial cells are a wildly used "helper" cell for supporting early stage embryos to past the in vitro developmental block (see reviews, Rexroad, 1989; Thibodeaux and Godke, 1992). Positive results have been reported with oviduct cell monolayers in sheep (Rexroad and Powell, 1986; Gandolfi and Moor, 1987), cattle (Eyestone and First, 1989; Ellington et al., 1990b) and pig (White et al., 1989). However, in the present study, no significant improvement on blastocyst development was detected using oviduct cell co-culture compared with medium alone culture when TCM-199 plus 5% FBS was used as culture medium. It has been proposed that helper cells benefit early embryos developing in vitro by producing an embryotrophic factor(s) or removing toxic substances from the culture medium (Kane et al., 1992). In this experiment, oviduct cells used with complex serum-containing medium (TCM-199 plus 5% FBS) seems not as effective as those used with simple serum-free medium (CZB) on promoting embryo development in vitro. The reason for this difference is not known.

Controversial results with the use of bovine oviduct cell co-culture have also been reported previously. Aoyagi et al. (1990) reported that bovine oviduct cells were more effective for stimulating blastocyst development of IVF-derived early stage bovine embryos in vitro than bovine cumulus cells. Whereas, Berg and Brem (1990) reported that significantly more bovine IVF embryos developed to morula and blastocyst stages
with cumulus cell co-culture (32%) compared with oviduct cell co-culture (17%). Eyestone and First (1989) reported that both oviduct cell co-culture and oviduct cell conditioned medium culture enhanced the development of IVF-derived embryos to the morula and blastocyst stages (22% and 22%, respectively). However, Ellington et al. (1990b) reported that better embryo quality (as evidenced by the percentage of poor-quality nuclei number of cells per embryo and embryo quality score) and greater hatching rates resulted with fresh oviduct cell co-culture compared with frozen-thawed oviduct cells and conditioned medium when culturing 1- to 2-cell stage bovine embryos in vitro. Bavister et al. (1992) also reported that the bovine oviduct cell conditioned medium did not increase morula and blastocyst formation compared with medium alone. A possible explanation for these controversial results may be that the stage of the bovine estrous cycle has an effect on the performance of oviduct cells during co-culture. In the present study, oviducts were harvested from the animals at midcycle (presence of luteal tissue). Xu et al. (1992a) reported that co-culture with bovine oviduct cells harvested from preovulatory (presence of a corpus haemorrhagicum or a mature follicle in the absence of corpus luteum) animal significantly improved the in vitro development of IVF-derived bovine embryos compared with medium alone. Further study is needed to investigate the cell source, culture condition, as well as the mechanism of the beneficial role of oviduct cell on embryo development.

When CZB medium (without serum) was compared with TCM-199 (with serum), significant more embryos developed to morula stage in CZB than in TCM-199. However, significant fewer embryos hatched in vitro in CZB than TCM-199. This indicates that fetal bovine serum inhibited embryo cleavage at early development stages
but stimulated \textit{in vitro} development at later stages, especially near the time of hatching. This developmental pattern on \textit{in vitro} development of IVF-derived bovine embryos with bovine calf serum has also been reported by Pinyopummintr and Bavister (1991). They indicated that the inhibition effect of serum was at or before the first cleavage division. In our study, however, only 2- to 8-cell stage embryos were used, and significantly fewer embryos developed to compacted morula stage when serum was present, which indicates the inhibition effect of the serum on early stage bovine embryo development was not only at first cell division but also several subsequent cell divisions and blastomere compaction. At the embryo hatching stage, significant improvement was observed when serum was present in the culture system, which is in agreement with the results reported by Pinyopummintr and Bavister (1991). Although the mechanism of this biphasic effect of serum on bovine embryo development remains unclear, this finding suggests that a culture system that starts with serum-free medium and then systematically increases the serum concentration of the medium to fit the embryo developmental stages may be more suitable for \textit{in vitro} producing bovine embryos.

In this study, there was a marked reduction in the number of morulae that developed into blastocysts during \textit{in vitro} culture in all treatment groups. This result suggests there may be another \textit{in vitro} development block at morula to blastocyst transition in addition to the 8- to 16-cell \textit{in vitro} block in bovine prehatched embryos. If this is the case, morula stage development \textit{in vitro} may not be a good indicator for embryo culture effectiveness. Further studies are needed to verify the existence of a secondary "block" and to further develop efficient \textit{in vitro} culture systems for bovine embryos.
CHAPTER 7

EFFECT OF GROWTH FACTORS AND GROWTH MODULATORS ON BOVINE OOCYTE IVM/IVF/IVC

Introduction

The regulatory effect of peptide growth factors on somatic cell proliferation and differentiation during in vitro culture has been well established (see review by Gospodarowicz and Moran, 1976). However, how growth factors affect the development of in vitro cultured early stage mammalian embryos remains to be determined. Although in some species, such as mouse (Whitten, 1956; Cholewa and Whitten, 1970), rabbit (Kane and Foote, 1970; Kane, 1972) and human (Caro and Trounson, 1986), early stage (1- to 2-cell) embryos can develop to blastocysts and hatch in defined culture medium without the addition of growth factors, it has become evident that early embryos may require endogenous as well as exogenous growth factors for normal development.

First, in vitro culture system have been shown not to be as efficient as in vivo on the development of early stage mammalian embryos. In vitro developmental block has been reported in several mammalian species, including hamster at 2- to 4-cell stage (Whittingham and Bavister, 1974), rat (Bavister, 1988) and pig (Davis and Day, 1978) at 4- to 8-cell stage, and sheep (Wintenberger et al., 1953), goat (Betteridge, 1977) and cow (Thibault, 1966) at 8- to 16-cell stage. Bowman and McLaren (1970) studied the growth rate of in vitro and in vivo developed mouse embryos and found that the cleavage rates of in vitro developed embryos were slower than that of in vivo developed embryos. Iwasaki et al. (1990) reported that at the same developmental stages, the cell number of in vitro cultured IVF-derived bovine embryos was lower than that of in vivo cultured
IVF-derived embryos and in vivo produced embryos. However, no difference was found between in vivo cultured IVF-derived embryos and in vivo produced embryos. These findings suggest that some paracrine factor(s) from the oviduct was involved in the early stage embryo development, and this factor(s) was missing in the in vitro culture systems.

Several growth factors, such as epidermal growth factor (EGF), transforming growth factor (TGF)-α, TGF-β1, colony-stimulating factor (CSF)-1, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-I, IGF-II, fibroblast growth factor (FGF) and interferon-γ have been detected in human uterine endometrium (see review by Giudice, 1994). Transcripts encoding for IGF-I, IGF-II, TGF-α, TGF-β1, TGF-β2, bFGF, PDGF-A and the receptors for IGF-I, IGF-II, insulin and PDGF-α have also been detected in ovine and bovine oviductal epithelial cell cultures (Watson et al., 1994), which may explain the supportive effect of oviduct cell co-culture on early embryo development.

Secondly, improved in vitro development of mouse embryos has been reported by decreasing the volume of culture medium or group culturing the embryos (Paria and Dey, 1990). This finding suggests that autocrine factor(s) may also participated in early embryo development. Recent studies have shown that the early stage mammalian embryos were able to produce a number of growth factors. Rappolee et al. (1988) reported the expression of PDGF, TGF-α, and TGF-β1 in mouse embryos. Zhang et al. (1994) detected the gene expression of IGF-I, IGF-II, IGF-I receptor, IGF-II receptor and insulin receptor in rat prehatched embryos. The expression of similar growth factor ligand and receptor genes have also been reported in cow embryos (Watson et al., 1992), sheep embryos (Watson et al., 1994) and human embryos (Schultz and Heyner).
The results of the experiments in which growth factors were applied exogenously to cultured embryos suggest that some growth factors may facilitate early embryo development in vitro. Paria and Dey (1990) reported that the addition of EGF or TGF-α or TGF-β1 to the culture medium markedly improved the development of single cultured mouse embryos. Harvey and Kaye (1992) reported that IGF-I increased the number of cells in the inner cell mass (ICM) of in vitro cultured mouse blastocysts when added to the medium for culturing 2-cell stage mouse embryos. The in vitro development of rat (Zhang and Armstrong, 1990) embryos and IVF-derived bovine embryos (Harper and Brackett, 1993) were also enhanced by adding growth factors to culture media.

The objective of this study was to evaluate growth factors and growth modulators on in vitro maturation (IVM), in vitro fertilization (IVF) and subsequent embryo development during in vitro culture (IVC) of bovine oocytes by adding purified products to culture medium. These include: insulin, growth hormone (GH), EGF, FGF, IGF-1, PDGF, prostaglandin F₂α (PGF₂α) and arachidonic acid (AA).

Materials and Methods

Ovary collection and oocyte aspiration

Ovaries were collected from mixed breed cows or heifers at a local abattoir immediately after slaughter. Excised ovaries were placed in physiological saline (0.9% NaCl) containing antibiotics (100 IU penicillin, 100 μg streptomycin/ml) and maintained at 25 to 28°C during transportation to the embryo laboratory. In the laboratory, ovaries were rinsed three times in saline and dried with a sterile paper towel. Oocytes were aspirated from small antral follicles (2 to 8 mm in diameter) using an 18-gauge needle attached to a 6 ml sterile, plastic syringe. Follicular fluid was placed in a 50-ml conical
After a sedimentation interval (3-5 minutes), supernatant was discarded and 10 ml of PBS with .1% PVA was added to the centrifuge tube. The oocyte-PBS suspension was poured into a 100 mm sterile plastic petri dish for oocyte searching and evaluation. Only those oocytes with intact cumulus and homogeneous cytoplasm were used in this study. Ovary washing and oocyte collecting were performed at room temperature (25 to 28°C).

**Experimental design**

**Experiment 7.1. Effect of insulin on bovine IVM/IVF/IVC**

This experiment was designed to evaluate the effect of insulin on bovine oocytes in vitro maturation (IVM), in vitro fertilization (IVF) and subsequent embryo development during in vitro culture (IVC). Insulin was added into culture medium during oocyte maturation incubation. The culture medium used in this study was Tissue Culture Medium 199 (TCM-199) supplemented with 5% fetal bovine serum (FBS), which is the culture medium used in the standard bovine IVF protocol (see Chapter 3). The insulin concentration was .1 μg/ml. Selected oocytes (n=800) were randomly assigned to two treatment groups. Oocytes were either cultured in the medium containing insulin or in medium alone (control) for in vitro maturation. Cumulus cell expansion scores were recorded after maturation incubation. A score of 1 to 4 was assigned to each oocyte: 1) cumulus cells had almost no expansion; 2) cumulus cells had moderate expansion, corona radiata cells had no expansion; 3) cumulus cells were fully expanded, corona radiata cells had no expansion; and 4) both cumulus cells and corona radiata cells were fully expanded.
Matured oocytes were inseminated with Ca\textsuperscript{++} ionophore A23187 treated sperm cells according to the standard IVF protocol (Chapter 3). The cleaved embryos were then co-cultured on cumulus cell monolayers for 8 days for \textit{in vitro} development. The embryo development culture medium was TCM-199 plus 5\% FBS. The proportion of oocytes matured, fertilized, cleaved and subsequently developed to morula, blastocyst, and hatched blastocyst stages were recorded. By day-8 of co-culture, embryos that developed to morulae, blastocysts and hatched blastocysts were fixed and stained with Hoechst stain 33342 for cell count. The experimental design of this experiment is outlined in Table 15.

**Experiment 7.2. Effect of GH, EGF, FGF, IGF-I and PGF\textsubscript{2\alpha} on embryo development**

This experiment was designed in a 6 x 2 factorial arrangement. The effect of the five growth factors (GH, EGF, FGF, IGF-I and PGF\textsubscript{2\alpha}), fetal bovine serum (FBS) and interactions were investigated. Oocytes were randomly assigned to each of 12 treatment groups for maturation, fertilization and embryo development culture. Embryo development was supported with cumulus cell co-culture. The growth factors were added to culture medium throughout the \textit{in vitro} culture interval. The basal medium used in this experiment was TCM-199 supplemented with .1\% polyvinyl alcohol (PVA). The concentration of growth factors are: 50 ng/ml GH, 10 ng/ml EGF, 50 ng/ml FGF, 100 ng/ml IGF-I and 50 ng/ml PGF\textsubscript{2\alpha}. Each growth factor was added into either the basal culture medium or the basal culture medium plus 5\% FBS. The experimental outline is shown in Table 16.
Table 15. Experimental design for Experiment 7.1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin*</td>
<td>400</td>
</tr>
<tr>
<td>Control</td>
<td>400</td>
</tr>
</tbody>
</table>

*Insulin concentration was .1 $\mu$g/ml of medium.
Table 16. Experimental design for Experiment 7.2

<table>
<thead>
<tr>
<th>Growth Factor*</th>
<th>No. of oocytes with FBS</th>
<th>No. of oocytes without FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>EGF</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>FGF</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>IGF-1</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Control</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

* GH = growth hormone; EGF = epidermal growth factor; FGF = fibroblast growth factor; IGF-1 = insulin-like growth factor-I; PGF$_{2\alpha}$ = prostaglandin F$_{2\alpha}$. 
Experiment 7.3. Effect of AA and PDGF on IVF embryo development

Experiment 7.3a. This experiment was designed to evaluate the effects of arachidonic acid (AA) and platelet-derived growth factor (PDGF) on bovine oocyte in vitro maturation, fertilization and subsequent development. Oocytes were cultured in the basal culture medium (TCM-199 plus .1% PVA) containing: 1) 50 ng/ml AA; 2) 5 ng/ml PDGF; 3) 5% FBS; and 4) basal medium alone for in vitro maturation, fertilization and early development. Cleaved embryos were then randomly divided into two subgroups. They were cultured continuously in the same medium alone or co-cultured on a cumulus cell monolayer to test if the presence of somatic cells is necessary for embryos to utilize AA and PDGF. The cumulus cell monolayers were developed from the cumulus cells that attached to the bottom of culture plates during oocyte maturation and fertilization. The experimental design is shown in Table 17.

Experiment 7.3b. Based on the result of Experiment 7.3a, adding AA into culture medium had significant effect on blastocyst formation, expansion and hatching compared with other treatment groups in both co-culture and medium alone culture. This effect was equivalent to FBS when embryos were cultured in medium alone, and was greater than the presence of FBS when embryos were co-cultured with cumulus cells. However, adding PDGF had no effect at all. In Experiment 7.3b, the effect of AA on embryo development was further investigated. AA was added into the culture medium 1) for oocyte maturation; 2) for embryo development; 3) throughout in vitro culture period; and 4) no AA addition as control. The cleaved embryos were subsequently co-cultured on cumulus cell monolayer as in Experiment 7.3a. The uncleaved oocytes were fixed and stained to examine the arrested stages. The experimental outline is shown in Table 18.
Table 17. Experimental design for Experiment 7.3a

<table>
<thead>
<tr>
<th>Treatment groups*</th>
<th>No. of oocytes</th>
<th>Co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>200</td>
<td>+</td>
</tr>
<tr>
<td>PDGF</td>
<td>200</td>
<td>+</td>
</tr>
<tr>
<td>FBS</td>
<td>200</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>200</td>
<td>+</td>
</tr>
</tbody>
</table>

*AA = arachidonic acid; PDGF = platelet-derived growth factor; FBS = fetal bovine serum.
Table 18. Experimental design for Experiment 7.3b

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>AA*</th>
<th>No. of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maturation</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Development</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Throughout</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

*AA = arachidonic acid (50 ng/ml added to the medium).
Experiment 7.3c. Based on the result of Experiment 7.3b, adding AA into culture medium during early embryo development had significant stimulating effect at expanded blastocyst and hatched blastocyst stages. In Experiment 7.3c, the dose response of AA on the development of IVF-derived embryos was tested. Two- to 8-cell stage IVF-derived embryos were cultured using basal culture medium supplemented with 1) 0 ng/ml AA (control); 2) 10 ng/ml AA; 3) 50 ng/ml AA; 4) 100 ng/ml AA; and 5) 500 ng/ml AA. Cumulus cell co-culture system was used in this experiment. The experimental design outline is shown in Table 19.

Source of growth factors

The peptide growth factors, including EGF, FGF, IGF-1 were obtained from Collaborative Research Incorporated. Insulin, GH, PDGF, PGF$_{2\alpha}$, and AA were purchased from Sigma. Tissue Culture Medium 199 (TCM-199) (25 mM HEPES, Earle’s salts), Dulbecco’s phosphate buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Gibco. Bovine serum albumin (BSA, fraction V), Polyvinyl alcohol (PVA) and other chemicals were obtained from sigma. All reagents used in this study were cell culture tested.

Oocyte in vitro maturation and fertilization

The in vitro maturation (IVM), in vitro fertilization (IVF) procedure used in this study was the same as described previously (Chapter 3). Briefly, selected oocytes were washed two times in PBS containing .1% PVA, two times in culture medium, and placed into four-well tissue culture plate containing 500 $\mu$l of culture medium in each well (20-25 oocyte/well). The basal culture medium used in this study was TCM 199
Table 19. Experimental design for Experiment 7.3c

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of 2 to 8-cell embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ng/ml AA*</td>
<td>100</td>
</tr>
<tr>
<td>10 ng/ml AA</td>
<td>100</td>
</tr>
<tr>
<td>50 ng/ml AA</td>
<td>100</td>
</tr>
<tr>
<td>100 ng/ml AA</td>
<td>100</td>
</tr>
<tr>
<td>500 ng/ml AA</td>
<td>100</td>
</tr>
</tbody>
</table>

*AA = arachidonic acid.
plus .1% PVA or TCM 199 plus 5% FBS (Experiment 7.1). Oocytes were incubated 22 to 24 h for maturation at 39°C in 5% CO₂ in humidified air.

At 1 h prior to insemination, two straws of frozen semen from a fertile Holstein dairy bull were thawed in a 25°C water bath for 1 min. The sperm cells were washed twice in B-O medium (Brackett and Oliphant, 1975) supplemented with 10 mM caffeine by centrifugation (350 x g, 6 min), and adjusted to a concentration of 3 x 10⁶ motile sperm cells/ml. Sperm cells were then exposed to Ca²⁺ ionophore A23187 (final ionophore concentration of .1 μM) for 1 min to aid in capacitation. Approximately 50 μl of treated sperm cell suspension were then added to each 50 μl fertilization droplet containing 20 to 25 oocytes. The fertilization medium was B-O medium containing 20 mg/ml BSA. Oocytes and sperm cells were co-incubated at 39°C in 5% CO₂ in humidified air for 6 h.

Embryo culture and evaluation

After 6 h of incubation, oocytes with attached cumulus cells were washed with culture medium and transferred either in 500 μl of culture medium in four-well tissue culture plate (25 oocytes/well) or into 100 μl of culture medium in 8 x 12-well tissue culture plate (10 oocytes/well). Oocytes were then cultured for an additional 43 h to evaluate the rate of cleavage in vitro. At the time of cleavage evaluation, cumulus cells surrounding the embryos were removed manually with a small pore glass pipette. The cells that already attached to the bottom of the culture plate were maintained as co-culture feeder cells. The cleaved embryos were subsequently cultured on cumulus cell monolayer or in medium alone at 39°C in 5% CO₂ in air until reached the hatched blastocyst stage. The culture medium was replaced with fresh medium at a 48-h
intervals, and the embryo development was recorded immediately after medium change. Uncleaved oocytes were fixed at 48 h post-insemination in ethanol:acetic acid (3:1) for 24 h, and stained with 2% aceto-orcein to examine the arrested stages.

Statistical analysis

For those experiments with factorial arrangement, the results were analyzed with SAS categorical data modeling (CATMOD) procedure (SAS Institute, Gary, NC), and the logit model was used to fit the observed data. For single factor designed experiments, Chi-square analysis was used to test the difference in the proportion of oocytes matured, fertilized and developed to certain developmental stages among treatment groups. The cumulus cell expansion score and cell count were analyzed using Student-t test. A P-value of < .05 was regarded as statistically significant.

Results

Experiment 7.1

In this experiment, the effect of adding insulin to culture medium during oocyte maturation incubation on bovine oocyte IVM/IVF/IVC was evaluated. The results of cumulus cell expansion score, the proportion of oocyte matured, fertilized and developed to various stages, as well as, the cell count (Figure 4) for morula, blastocyst and hatched blastocyst stage embryos are shown in Table 20. Insulin addition had a significant effect on cumulus cell expansion. The mean cumulus cell expansion score in insulin treated group was 3.61, which was significant higher than score of 3.20 from control group (P < .05). The fertilization rate was also significantly greater for the insulin treated group compare with the control group (P < .05). There were no detectable difference between two treatment groups on the proportion of oocytes that cleaved, and
Figure 4. A blastocyst (a) and a hatched blastocyst (b) developed from insulin treatment group after Hoechst 33342 staining of the nuclei. The appearance of meiotic figures and the lack of fragmented nuclei were suggestive of healthy viable embryo. Magnification: X400.
Table 20. Bovine oocytes matured *in vitro* with and without insulin*  

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of oocytes</th>
<th>CCE score</th>
<th>FERT %</th>
<th>CLEAV %</th>
<th>MORL % (cell no.)</th>
<th>BLST % (cell no.)</th>
<th>HBLST % (cell no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>411</td>
<td>3.20a</td>
<td>66a</td>
<td>54</td>
<td>41 (34)a</td>
<td>27 (75)</td>
<td>19 (509)</td>
</tr>
<tr>
<td>Insulin</td>
<td>401</td>
<td>3.61b</td>
<td>77b</td>
<td>55</td>
<td>42 (49)b</td>
<td>23 (93)</td>
<td>17 (888)</td>
</tr>
</tbody>
</table>

*Abbreviations: CCE=cumulus cell expansion; FERT=fertilized; CLEAV=cleaved; MORL=morula; BLST=blastocyst; HBLST=hatched blastocyst.

*a,b Values in the same column with different superscripts are significantly different (P<.05).*
subsequently developed to morula, blastocyst and hatched blastocyst stages. However, the number of cells per embryo was significantly different at morula stage. Embryos developed from insulin treatment group had greater cell number per embryo than control group (P < .05). A similar trend was noted at blastocyst and hatched blastocyst stages, but there were no statistically different, due to a large standard deviation.

**Experiment 7.2**

The results of Experiment 7.2 are presented in Table 21. All growth factors tested in this experiment including growth hormone, epidermal growth factor, fibroblast growth factor, insulin-like growth factor-I, and prostaglandin F₂α had no effect on IVM/IVF/IVC of bovine oocytes in both serum-free medium and serum-containing medium compared with control (P > .05). When compare serum-containing and serum-free treatment effect, overall development rates were the same except serum-containing groups had higher cumulus cell expansion score (3.74 vs. 3.20) and fertilization rate (90.8% vs. 86.4%) (P < .05). Among treatment groups, 83 to 95% oocytes fertilized, 68 to 77% cleaved, 38 to 55% developed to morulae, 9.4 to 20.4% developed to expanded blastocyst and 4.7 to 12.4% developed to hatched blastocyst stage.

**Experiment 7.3a**

In this experiment, the effects of AA, PDGF and FBS on bovine oocyte IVM/IVF/IVC were tested. Cleaved embryos were either co-cultured on cumulus cell monolayer or cultured in medium alone. A summary of the results are given in Table 22. Overall, growth factor addition had a significant positive treatment effect (P < .001) on embryo development, which is due to the effect of AA according to the analysis of weighted-least-square estimates. Culture condition also had significant effect (P < .0005),
Table 21. Effect of growth factors on IVF-derived bovine embryos

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>No. of oocytes</th>
<th>CCES</th>
<th>FERT</th>
<th>CLEAV</th>
<th>MORL</th>
<th>ExBLAST</th>
<th>HBLAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH + 200</td>
<td>3.82</td>
<td>95.1</td>
<td>73.7</td>
<td>38.4</td>
<td>10.6</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>GH - 200</td>
<td>3.40</td>
<td>94.7</td>
<td>76.0</td>
<td>50.0</td>
<td>13.2</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>EGF + 200</td>
<td>3.71</td>
<td>87.8</td>
<td>76.9</td>
<td>52.3</td>
<td>13.3</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>EGF - 200</td>
<td>3.31</td>
<td>83.0</td>
<td>71.6</td>
<td>40.0</td>
<td>9.4</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>FGF + 200</td>
<td>3.69</td>
<td>90.2</td>
<td>69.2</td>
<td>39.5</td>
<td>14.3</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>FGF - 200</td>
<td>3.09</td>
<td>87.6</td>
<td>71.4</td>
<td>44.4</td>
<td>13.2</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>IGF-I + 200</td>
<td>3.66</td>
<td>86.8</td>
<td>69.0</td>
<td>42.3</td>
<td>14.2</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>IGF-I - 200</td>
<td>3.01</td>
<td>84.8</td>
<td>68.7</td>
<td>40.3</td>
<td>14.9</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>PGF2α + 200</td>
<td>3.79</td>
<td>93.2</td>
<td>72.6</td>
<td>49.7</td>
<td>13.7</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>PGF2α - 200</td>
<td>3.20</td>
<td>81.5</td>
<td>68.0</td>
<td>55.0</td>
<td>10.1</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>Control + 200</td>
<td>3.81</td>
<td>92.0</td>
<td>77.7</td>
<td>47.5</td>
<td>19.3</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>Control - 200</td>
<td>3.22</td>
<td>86.5</td>
<td>76.7</td>
<td>52.2</td>
<td>20.4</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Total + 1,200</td>
<td>3.74</td>
<td>90.8*</td>
<td>73.7</td>
<td>44.7</td>
<td>14.2</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>Total - 1,200</td>
<td>3.20*</td>
<td>86.4*</td>
<td>72.0</td>
<td>46.6</td>
<td>13.4</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: GH=growth hormone (50 ng/ml); EGF=epidermal growth factor (10 ng/ml); FGF=fibroblast growth factor (50 ng/ml); IGF-1=insulin-like growth factor-1 (100 ng/ml); PGF2α=prostaglandin F2α (50 ng/ml); FBS=fetal bovine serum (5%); CCES=cumulus cell expansion score; FERT=fertilized; CLEAV=cleaved; MORL=morula; ExBLST=expanded blastocyst; HBLST=hatched blastocyst.

* Values in the same column with different superscripts are significantly different (P < .05).
Table 22. Arachidonic acid and PDGF on bovine oocyte *in vitro* maturation, fertilization and development

<table>
<thead>
<tr>
<th>Trt group culture</th>
<th>Co-</th>
<th>No. of MATR</th>
<th>FERT</th>
<th>CLEAV</th>
<th>No. of MORL</th>
<th>BLAST</th>
<th>ExBLAST</th>
<th>HBLAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>+</td>
<td>224</td>
<td>93</td>
<td>83</td>
<td>81b</td>
<td>75</td>
<td>72</td>
<td>28</td>
</tr>
<tr>
<td>PDGF</td>
<td>+</td>
<td>205</td>
<td>90</td>
<td>80</td>
<td>75ab</td>
<td>62</td>
<td>73</td>
<td>18</td>
</tr>
<tr>
<td>FBS</td>
<td>+</td>
<td>229</td>
<td>92</td>
<td>83</td>
<td>71*</td>
<td>52</td>
<td>65</td>
<td>21</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>209</td>
<td>92</td>
<td>82</td>
<td>75ab</td>
<td>58</td>
<td>71</td>
<td>22</td>
</tr>
<tr>
<td>AA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>75</td>
<td>76b</td>
<td>23b</td>
</tr>
<tr>
<td>PDGF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>62</td>
<td>45*</td>
<td>8*</td>
</tr>
<tr>
<td>FBS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>52</td>
<td>65b</td>
<td>29b</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>58</td>
<td>48*</td>
<td>9*</td>
</tr>
</tbody>
</table>

*Abbreviations: Trt=treatment; AA=arachidonic acid (50 ng/ml); PDGF=platelet-derived growth factor (5 ng/ml); FBS=fetal bovine serum (5%). MATR=matured; FERT=fertilized; CLEAV=cleaved; MORL=morula; BLAST=blastocyst; ExBLAST=expanded blastocyst; HBLST=hatched blastocyst.*

*Values in the same column with different superscripts are significantly different (P < .05).*
more embryos developing to the later developmental stages when co-cultured with cumulus cells. There was an interaction between growth factor effect and embryo developmental stages, which indicates that the growth factor had a stronger stimulating effect at later developmental stages.

There were no differences on oocyte maturation rates and fertilization rates among four treatment groups. However, more oocytes cleaved from AA group compared with FBS group (P < .05), and no detectable difference either between AA and control or FBS and control groups. Pairwise comparison within each developmental stage showed that significant more embryos developed to hatched blastocyst stage from AA group compared with other treatment groups when embryos were co-cultured (P < .05), and significantly more embryos developed to morula, blastocyst and expanded blastocyst stages from AA and FBS groups compared with other two treatment groups when embryos were cultured in medium alone (P < .05).

**Experiment 7.3b**

This experiment evaluated the effect of adding AA at different *in vitro* culture intervals on bovine oocyte IVM/IVF/IVC (Table 23). AA was added into the basal culture medium during 1) maturation incubation, 2) embryo development incubation, 3) throughout *in vitro* culture period, and 4) no AA addition as a control. The rates of oocyte maturation, fertilization and cleavage were not affected by adding AA at all different culture periods compared with the control (P > .05). However, there was a trend that more embryos developed to expanded blastocysts and hatched blastocysts when AA was present in the culture medium compared with the control, although significant
Table 23. Adding AA at different time intervals on bovine oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of group oocytes</th>
<th>MATR</th>
<th>FERT</th>
<th>CLEAV</th>
<th>MORL/ CLEAV</th>
<th>BLAST/ CLEAV</th>
<th>ExBLAST/ CLEAV</th>
<th>HBLAST/ CLEAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maturation</td>
<td>108</td>
<td>86</td>
<td>34</td>
<td>30</td>
<td>63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53</td>
<td>53&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Development</td>
<td>108</td>
<td>91</td>
<td>35</td>
<td>27</td>
<td>69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59</td>
<td>59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Throughout</td>
<td>108</td>
<td>94</td>
<td>37</td>
<td>34</td>
<td>92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46</td>
<td>46&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>24&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>108</td>
<td>93</td>
<td>38</td>
<td>29</td>
<td>74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42</td>
<td>32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Abbreviations: AA=arachidonic acid (50 μg/ml); MART=matured; FERT=fertilized; CLEAV=cleaved; MORL=morula; BLST=blastocyst; ExBLST=expanded blastocyst; HBLST=hatched blastocyst.

<sup>a,b</sup>Values in the same column with different superscripts are significantly different (P < .05).
difference was detected only between adding AA during embryo development culture and the control (P < .05).

When AA was added throughout the whole in vitro culture period, more cleaved embryos developed to morula stage than that of other AA treatment groups and control group (P < .05). No significant difference were found among three AA addition groups at blastocyst, expanded blastocyst and hatched blastocyst stages (P > .05).

**Experiment 7.3c**

This experiment was to investigate the dose response of adding AA on the development of IVF-derived bovine embryos. Four concentration levels of AA (10, 50, 100 and 500 ng/ml) were evaluated in this experiment. AA was added to culture medium during embryo development culture. Two- to 8-cell stage IVF-derived embryos were randomly assigned to each treatment groups. Overall, adding 10 ng/ml, 50 ng/ml, 100 ng/ml, but 500 ng/ml of AA to the culture medium had a beneficial effect on in vitro embryo development (Table 24). The difference among treatment groups started to show up at blastocyst stage. More embryos developed to the blastocysts from 50 ng/ml group compared with the control (P < .05), and no difference was detected at this stage among 10 ng/ml, 50 ng/ml, and 100 ng/ml groups (P > .05). At expanded blastocyst and hatched blastocyst stages, the embryo development were significantly improved in 10 ng/ml, 50 ng/ml and 100 ng/ml groups compared with the control group (P < .05).

The AA concentration of 500 ng/ml had no effect on embryo development at all stages evaluated in this study compared with the control (P > .05). The treatment group that had the highest developmental rates at various stages was 50 ng/ml group,
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of 2 to 8 cell</th>
<th>No. of MORL (%)</th>
<th>No. of BLAST (%)</th>
<th>No. of ExBLAST (%)</th>
<th>No. of HBLAST (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ng/ml</td>
<td>96</td>
<td>61 (64)</td>
<td>30 (31) &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>25 (26) &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2 (2) &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>96</td>
<td>64 (67)</td>
<td>40 (42) &lt;sup&gt;bc&lt;/sup&gt;</td>
<td>27 (28) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>9 (9) &lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>96</td>
<td>70 (73)</td>
<td>43 (45) &lt;sup&gt;c&lt;/sup&gt;</td>
<td>35 (36) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>15 (16) &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>96</td>
<td>67 (70)</td>
<td>38 (40) &lt;sup&gt;bc&lt;/sup&gt;</td>
<td>29 (30) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 (10) &lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>500 ng/ml</td>
<td>96</td>
<td>70 (73)</td>
<td>26 (27) &lt;sup&gt;a&lt;/sup&gt;</td>
<td>16 (17) &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 (4) &lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Abbreviations: AA=arachidonic acid; MORL=morula; BLST=blastocyst; ExBLST=expanded blastocyst; HBLST=hatched blastocyst.

<sup>a,b,c</sup>Values in the same column with different superscripts are significantly different (P < .05).
with the development rates at blastocyst, expanded blastocyst and hatched blastocyst stages of 45, 36 and 16%, respectively.

Discussion

The role of peptide growth factor on preimplantation embryo development has attracted a great attention to the developmental biologists in recent years. The biological effect of growth factors have been demonstrated in intact animals, organ cultures and cell cultures (Gospodrowicz and Moran, 1976). However, how growth factor affect fertilization and early embryo development is unclear. The development of in vitro embryo culture systems and the success on in vitro fertilization in many mammalian species make it possible to investigate the effect of growth factor on early development in vitro. One approach to study growth factor on early development is by adding growth factors to culture media and measuring embryo developmental rate in culture. This approach may give a clue for further biochemical and molecular studies on growth factors and their receptors gene expression and protein synthesis by early embryos. It may also give some information that helps to reveal the growth factor reaction passway.

In the present study, this approach was used to evaluate six common peptide growth factors (insulin, GH, EGF, FGF, IGF-1, PDGF) and two fatty acids (PGF\textsubscript{2\alpha}, AA) on bovine oocyte in vitro maturation, fertilization and subsequent embryo development.

The addition of insulin

The data from present study demonstrated that insulin had a significant effect on cumulus cell expansion during oocyte in vitro maturation. This finding suggests that insulin was able to stimulate the functional hyaluronic acid synthesis and secretion within the cumulus mass (Chen et al., 1993). It has been reported that in several species, the
optimal growth and expression of function of granulosa cells require the presence of insulin (Channing et al., 1976; Orly et al., 1980; May and Schonbery, 1981; Savion et al., 1981). In addition, the quality of cumulus cell expansion has been shown closely related to the success of in vitro fertilization in cattle (Ball et al., 1983) and mouse (Chen et al., 1993). The results of present study are in agreement with those of previous findings that showed a significant improvement of in vitro fertilization in the insulin-treated oocyte compared with the control (P < .05). This evidence further demonstrates that insulin is able to modulate the metabolism and function of cumulus cells, which may affect the sperm penetration of the oocyte and subsequently fertilization.

Another interesting finding from this experiment was that although fertilization rate was higher in insulin treated group, the proportion of the oocytes that cleaved and developed to morula and blastocyst stages were not different. Furthermore, the number of cells per embryo was significantly greater in the insulin-treated group at morula stage (P < .05), and the same trend was found at blastocyst and hatched blastocyst stages. A possible explanation for these results is that at early maturation stage, insulin mainly associated with cumulus cell activity instead of oocyte itself. This causes cumulus cell expansion and facilitates sperm penetration, but does not have an effect on early embryonic cell division. After maternal-embryonic genomic transition, which has been proved at 8 to 16-cell stage in bovine (King et al., 1989), the embryos start to synthesize proteins and peptides that required for cell proliferation and differentiation. Insulin may be taken up by embryo and involved in the embryonic metabolism at this time. In contrast, insulin is a well known anabolic hormone, which promotes the synthesis of glycogen, protein, and lipid while inhibits the degradation of these substances (Czech,
1977). It have been reported that the protein, DNA and RNA synthesis in preimplantation mouse embryos were enhanced by insulin (Harvey and Kaye, 1988; Heyner et al., 1989). Therefore, in present study, the insulin supplementation of the culture medium may have had stimulatory effect on mitotic division of the embryonic cells after maternal-embryonic genomic transition and resulted in greater number of cells at morula and blastocyst stages.

One thing that should be mentioned in this study, is that insulin was added to the culture medium only during the time of oocyte maturation incubation, while the primary effects of insulin was on cell division at the morula and blastocyst stages. This may be an indication that insulin can be stored and then used by embryos. Correspondingly, Heyner et al. (1989) have reported that insulin receptors are expressed in early morula stage mouse embryos and increased in numbers through the blastocyst stages. Their study demonstrated that insulin was present in mouse oviductal fluid, and the insulin that was internalized by the early embryo was of maternal origin. They also reported that insulin could be absorbed nonspecifically in the zona pellucida and bound specifically to its receptors on cytoplasmic membrane of the blastomeres. The ability of zona pellucida to accumulate proteins was also reported by Schlafke and Enders (1973), which may explain the later on effect of insulin on embryonic cell division observed from the present study.

Cell number and the mitotic index of in vitro-cultured embryos are now used by developmental biologists as an indicator of embryo quality. It has been reported that in vivo-produced bovine embryos had greater cell number compared with in vitro-produced embryos at the morula blastocyst and expanded blastocyst stages (Iwasaki et al., 1990),
and in vivo-cultured IVF-derived embryos had similar cell number as in vivo produced embryos (Harlow and Quinn, 1982; Iwasaki and Nakahara, 1990). This finding indicates that existing in vitro culture system is not as efficient as in vivo, although oocytes can be matured, fertilized and cultured to morula, blastocyst, even hatched blastocyst stages in vitro.

The viability of in vitro-produced embryos may not be as good as in vivo-produced embryos when pregnancy after transfer to recipient animals is used as the indicator of embryo quality (Bowman and McLaren, 1970; Gandolfi and Moor, 1978). The present study showed a significant improvement in the number of cells per embryo by adding insulin into the maturation medium, which confirmed previous findings that insulin is involved in mammalian early stage embryo development by Heyner et al. (1988,1989). This further indicates that insulin may play a role in early bovine embryonic development.

The addition of GH, EGF, PGE, IGF-1, PGF2α.

Experiment 7.2 was designed to investigate the effect of primary growth factors on IVM/IVF/IVC of bovine oocytes. The only difference detected in the results was that the overall cumulus cell expansion scores and fertilization rates were greater when serum was presented in the culture medium (P < .05). This would suggest a positive effect of serum on cumulus cell expansion and sperm penetration. However, except cumulus cell expansion and fertilization rates, the proportion of oocyte matured, cleaved and developed to morula, blastocysts and hatched blastocysts were not different when compared with control medium either with or without serum supplementation. This findings suggest that serum may not be necessary for IVM/IVF/IVC of bovine oocytes.
Exogenous serum has long been used as a cell culture additive to stimulate cellular proliferation, cell attachment and biological production (Jayme et al., 1988). Beneficial effect of serum supplementation has been demonstrated in oocyte in vitro maturation, fertilization and subsequent embryo development (Choi et al., 1987; Vanderhyden and Armstrong, 1989; Leibfried-Rutledge et al., 1987). Serum has been used as a medium supplement in most bovine IVM/IVF/IVC systems with concentration in the media ranging from 5% (Goto et al., 1989; Zhang et al., 1993) to 20% (Younis and Brackett, 1991; Xu et al., 1992). However, serum is an undefined fluid that exhibits lot-to-lot variability in biochemical composition and culture performance (Art to Science in Tissue Culture Vol. 5:3-4, Hyclone Laboratories, 1986).

Efforts have been made to improve the culture system by adding growth factors (Paria and Dey, 1990; Das et al., 1991; Harvey and Kaye, 1992), gonadotrophins (Younis and Brackett, 1992) or both (Happer and Brackett, 1993) to the serum-free culture medium to stimulate oocyte and embryo in vitro development. The results of the present study indicate that when cumulus cells were used as feeder cells for co-culture, oocyte maturation and embryo development were not affected by the presence of serum. Similar results have been reported by Saeki et al. (1991), who reported successful oocyte maturation, fertilization and embryo development to blastocyst stage in cattle using serum-free medium and cumulus cell co-culture. This finding allows us to eliminate the time consuming work on serum lot screening and possible contamination of the culture system from serum. Removing the serum provides for a more defined culture system to study the mechanism of cumulus cell co-culture on early mammalian development.
Recent studies have shown the evidences of the involvement of growth factors in early mammalian embryonic development. The expression of growth factor genes (Rappolee et al., 1988), the presence of growth factor receptors (Rappolee et al., 1990; Adamson, 1990; Wiley et al., 1992) and the growth promoting effect of exogenous growth factors on mammalian oocyte and preimplantation embryo development (Yoshida and Kanagawa, 1984; Paria and Dey, 1990; Das et al., 1991; Harvey and Kaye, 1992; Harper and Brackett, 1993) have been reported. However, some research groups also reported the lack of effect of exogenous growth factors on bovine oocyte IVM/IVF/IVC (Colver et al., 1991; Flood et al., 1993).

The lack of effect of growth factors on oocytes maturation, fertilization and embryo development from the present study may be explained as follows. First, in present study, oocytes and embryos were cultured in groups (20 to 25/group in .5 ml culture medium) instead of individually. Therefore, the effect of exogenous growth factors could be covered by the effect of embryonic origin growth factors that participate in embryo development in an autocrine manner. Paria and Dey (1990) reported a cooperative interaction among embryos in culture, and indicated that this interaction is mediated by specific growth factors released by embryos. They found that 2-cell mouse embryos cultured singly had low development to blastocyst stage and lower cell number per blastocyst compared with those cultured together in groups. Their study also showed that the low development of single-cultured embryos was markedly improved by addition of EGF or TGF-β1 to the culture medium. The beneficial effect of group embryo culture has also been reported by Wiley et al. (1986).
Secondly, since the cumulus cell co-culture system was used in the present experiment, the effect of exogenous growth factors could be masked by the interaction between embryos and the helper cells. One hypotheses on the beneficial effect of co-culture is that helper cells may produce substance(s) that promote embryonic cell proliferation and differentiation (Kane et al., 1992). There is a wide variety of somatic cell types that have been shown to enhance embryo development \textit{in vitro} (Camous et al., 1984; Voelkel et al., 1985; Rexroad and Powell, 1986, Goto et al., 1989, Kim et al., 1991).

In summary, this experiment showed no effect of GH, EGF, FGF, IGF-1, and PGF$_{2\alpha}$ on \textit{in vitro} development of IVF-derived bovine embryos with or without serum supplementation under our current IVM/IVF/IVC system. This study also demonstrated that bovine oocytes can be matured, fertilized and cultured to hatched blastocyst in serum-free medium when co-cultured with cumulus cells originated from oocyte cumulus complex.

The addition of AA and PDGF

The effect of arachidonic acid and platelet derived growth factor were evaluated in Experiment 7.3a. Based on the finding of Experiment 7.2, the co-culture system may mask the effect of exogenous growth factors. In the present experiment, cleaved embryos from each treatment group were randomly divided into two groups and were either co-cultured with cumulus cells or cultured in medium alone. The major finding from this experiment was that the addition of AA to culture medium had significant effect on blastocyst hatching when embryos were co-cultured compared with other treatment groups. The proportion of embryos developed to morula, blastocyst and expanded
blastocyst were enhanced with AA addition when embryos were cultured in medium alone. This finding suggests that AA was able to promote bovine embryo development by affecting blastomere compaction, embryonic cell differentiation, blastocyst expansion and hatching. Furthermore, these results further verified that the effect of exogenous growth factors could be masked by co-culture. The results showed the equivalency of AA to cumulus cell co-culture and serum supplementation on morula, blastocyst formation and blastocyst expansion.

AA is a C20 essential fatty acid and a precursor of prostaglandins (PG). The uptake of AA by prehatched embryos (mainly blastocyst stage embryos) from the culture medium has been reported in several mammalian species including cattle (Menezo et al., 1982; Hwang et al., 1988), sheep (Marcus, 1981; Sayer and Lewis, 1993) and primates (Neulen et al., 1991). Furthermore, it has been shown AA can be incorporated into the prehatched embryos and converted inside the embryo into a number of compounds, mainly prostaglandin and thromboxanes (Hwang et al., 1988; Neulen et al., 1991; Sayre and Lewis, 1993). It is reported that the conversion of AA to PG by embryos may have the role in embryo hatching from the zona pellucida (Biggers et al., 1978; Chida et al., 1986; Sayre and Lewis, 1993). The results from the present study are further demonstrated a hatching promoting effect of AA on the development of in vitro cultured, IVF-derived bovine embryos both in co-culture and medium alone culture systems.

Beside hatching, the results of present study also provided an evidence that AA is able to stimulate embryo development at the stage of first cell division, morula and blastocyst formation. For many years, research had been focused on how AA convert to PG and then affect mammalian development. However, resent studies showed that AA
is directly involved in the signal transduction system that regulates the cell metabolism. It has been proposed that AA is a second messenger generated from membrane phospholipid, which activates the enzymes and regulates the ion channels (O'Neill et al., 1990; Hansel et al., 1991). Chien et al. (1986) reported that in progesterone induced amphibian oocyte maturation, AA incorporation was increased after an increased Ca\textsuperscript{2+} efflux. AA was found to be able to trigger starfish oocyte maturation, which is normally induced by nature hormone 1-methyladenine (Meijer et al., 1986).

Using radiolabeling technique, Waterman and Wall (1988) found that rabbit zygotes were able to concentrate AA to a 170 fold of the initial concentration in culture medium during 6 h of incubation. These findings indicate that AA could be involved in maturation, oocyte activation during fertilization and early embryo development. In these events, AA may have a direct effect on development as a second message instead of converting into PG in the cells. The evidence of the direct action of AA is that AA was shown to be able to activate enzymes and ion channels (O'Neill et al., 1990).

A study on luteal cell function by Lukaszewska and Hansel (1980) demonstrated that AA stimulate luteal cell secretory function by inducing oxytocin and progesterone release. They also showed that this stimulatory effect do not result from PG formation (Lafrance and Hansel, 1992). This is further evidence of second message role of AA in modulating cell function.

In Experiment 7.3b, AA was added into culture medium during maturation, embryo development, or throughout the whole \textit{in vitro} culture period. The result of this experiment showed significant improvement of blastocyst expansion in all AA treated groups compared with the control (P < .05). Also, there was no difference among AA
treatment groups, which indicated that AA can be taken up by oocyte, zygote or early stage embryos. The effect of AA appeared at blastocyst stages as the embryos were co-cultured with cumulus cells in this experiment.

In Experiment 7.3c, the dose response of AA on IVF-derived bovine embryo development was evaluated. The concentration of 10 ng/ml, 50 ng/ml and 100 ng/ml of AA had a significant promoting effect on blastocyst formation, blastocyst expansion and embryo hatching \( (P < .05) \). The concentration of 500 ng/ml AA tested in this experiment did not improve embryo development when compared with the control group. The developmental rates at blastocyst and expanded blastocyst stages were found to be even lower than that of control group. This finding suggests a high level of AA had a negative effect on embryo development in vitro. Excess AA in the culture system may not only be unable to stimulate but also inhibit the development by blocking or altering other metabolism passway. The best embryo development rates obtained from this experiment was the group with the addition of 50 ng/ml of AA to the culture medium.

In this study, no effect was found when PDGF was added to culture medium when compared with the control. PDGF is one of the growth factors that its transcripts encoding ligand and receptor were found in oocyte and embryos, therefore, is probably synthesized by prehatched embryos (Rappolee et al., 1988; Watson et al., 1992, 1994). It is proposed that PDGF may have effect on embryonic cell proliferation and differentiation. The possible explanation for the results of the present study could be that embryos do not need exogenous PDGF at early developmental stages. Secondly, PDGF of embryo origin is enough to support the early development. As mentioned earlier, the
cooperative interaction among embryos may exist in this study because embryos were cultured in groups.

This study showed a strong evidence of arachidonic acid stimulating embryo development *in vitro*. The positive effect was mainly on the development of morula, blastocyst and hatching stages. This effect was significant even when embryos were cultured together in group and co-cultured with somatic cells. Furthermore, these results indicate that AA can be taken up by oocytes, zygotes or early cleavage stage embryos from culture media. In this study, the optimal AA concentration was 50 ng/ml of medium; whereas, the 500 ng/ml concentration of AA appeared to be toxic to cultured embryos.

In conclusion, adding growth factor(s) into culture media is a useful approach to study the effect of growth factor on preimplantation embryo development. Previous studies provided the evidence of synthesizing and releasing growth factors by early embryos, and the presence of growth factor receptors on early embryos. The present study further demonstrated that insulin and arachidonic acid are involved in bovine oocyte maturation, fertilization and early embryo development. This study also showed the overlapping effect of growth factor with co-culture or the presence of serum in culture medium. Some growth factors tested in this study had no effect on early development, which may reflect the presence of cooperative interaction among those group cultured embryos in our culture system. Further study is needed to evaluate the growth factors on embryo *in vitro* development in a well controlled culture system. This is needed to reveal the regulating passway of growth factors on mammalian early development.
CHAPTER 8

BIRTH OF LIVE CALVES AFTER TRANSFER OF FROZEN-THAWED BOVINE EMBRYOS FERTILIZED IN VITRO

Introduction

The first successful in vitro fertilization procedure producing a live calf was reported in the USA in the early 1980s (Brackett et al., 1982a). Since then, the methods for in vitro maturation and fertilization have markedly advanced. Bovine morulae and blastocysts can now be obtained routinely from in vitro matured/in vitro fertilized and in vitro cultured oocytes collected from abattoir ovaries. Although pregnancies and live calves have been obtained from the transfer of embryos matured, fertilized and cultured in vitro in several countries (Goto et al., 1988; Lu et al., 1988; Xu et al., 1990; Jiang et al., 1991; Monson et al., 1992), the question remains whether in vitro fertilized embryos are as viable in utero as in vivo-derived embryos. Furthermore, it is not known whether bovine embryos fertilized in vitro can survive freezing and then produce viable offspring after being transferred into a recipient cow. Iwasaki et al. (1990) have reported that in vitro fertilized bovine embryos often have a smaller inner cell mass and fewer cells at the blastocyst stage than embryos of a similar age produced in vivo. In this case, culturing embryos fertilized in vitro could increase the chance of success after in vitro fertilization by transferring embryos that are more developed and from a more selected embryo population. If culturing an embryo after it has been thawed offers an advantage, then it should be considered for developing frozen-thawed in vitro fertilized embryos before they are transferred to recipient animals.
The objective of this study was to evaluate the viability of *in vitro* fertilized bovine embryos after freezing and thawing and their subsequent pregnancy rates in recipient cattle.

**Materials and Methods**

**Experimental oocytes**

Ovaries from beef and dairy cattle were collected at a local abattoir and transported to the laboratory in Dulbecco's-phosphate buffered saline (PBS) at 23 to 25°C. In the laboratory, ovaries were washed three times in PBS containing antibiotics (100 IU penicillin and 100 μg streptomycin/ml). The oocytes were aspirated from 3 to 8 mm follicles using an 18-gauge needle attached to a 6 ml sterile, plastic syringe. The oocytes were evaluated for the number of layers of cumulus cells and washed twice in PBS containing 0.1% polyvinyl alcohol (PVA). The oocytes with intact cumulus cells and homogenous cytoplasm were used in this study.

**In vitro maturation and fertilization procedures**

Good quality cumulus-intact oocytes were placed in four-well tissue culture plates (Nunclon) (25 oocytes/well) and incubated at 39°C for 24 h in 0.5 ml of Tissue Culture Medium 199 (TCM-199) supplemented with 5% fetal bovine serum (FBS) in 5% CO₂ in an atmosphere of air for maturation. Matured oocytes were then exposed to the standard bovine *in vitro* fertilization procedure used in this laboratory (Chapter 3).

Briefly, two straws of frozen semen from a fertile Holstein dairy bull were thawed in a 25°C water bath for 1 min. The sperm cells were washed in Brackett-Oliphant (B-O) medium supplemented with 10 mM caffeine and adjusted to a concentration of 3 x 10⁶ motile sperm cells/ml and then exposed to Ca++ ionophore
A23187 (ionophore concentration of .1 \( \mu M \)) for 1 min to aid in capacitation. Sperm and oocytes were then incubated in a 100 \( \mu l \) drop of B-O medium with 10 mg/ml bovine serum albumin (BSA) at 39°C in 5% \( CO_2 \) in an atmosphere of air. After 6 h of incubation, the oocytes (with their cumulus cells) were moved to TCM-199 with 5% FBS and cultured for an additional 43 h at 39°C in 5% \( CO_2 \) in air.

Cumulus cells surrounding the embryos were removed manually with a glass pipette. These cells and those already attaching to the culture dish were then maintained as a co-culture system. The fertilized embryos were then cultured with the attached cumulus cells in TCM-199 with 5% FBS at 39°C in 5% \( CO_2 \) in air. The culture medium was replaced with fresh medium at 48-h intervals. The embryos were evaluated immediately after the medium change using an inverted microscope to establish the development of morula, blastocyst and expanded blastocyst stages in vitro.

**Embryo freezing and thawing**

When 300 contemporary in vitro-fertilized embryos reached either the 16-cell, morula or early blastocyst stage they were placed in .25 ml plastic straws (2 embryos/straw) and frozen in 10% glycerol using a standard embryo freezing procedure (Leibo, 1986). The embryos were plunged into liquid nitrogen (-196°C) when they had reached -33°C. After approximately 1 year of storage in liquid nitrogen, 49 straws (containing 98 embryos at various morphological stages) were selected and thawed in air at room temperature (22°C). The cryoprotectant was removed in three 5-min steps with mixtures of sucrose and glycerol (.3 M sucrose with 10% glycerol, 5% glycerol and no glycerol) and then into PBS medium. The embryos were then evaluated in terms of their
morphological development and placed into in vitro culture treatment groups before being transferred to recipient females.

Post thaw embryo culture and transfer

Frozen-thawed embryos were cultured either for 4 h in TCM-199 or cultured for 2 days on cumulus cells with TCM-199, as previously described by Zhang et al. (1992b). Embryos from the group cultured for up to 4 h, which appeared viable, were then transferred nonsurgically to recipient females (1 or 2 embryos/female) ipsilateral to the corpus luteum on days 6 or 7 of their estrous cycle (estrus=day 0). Similarly, morulae and blastocysts from the group cultured for 2 days were then transferred to recipient animals (1 or 2 embryos/female) on days 7 or 8 of their estrous cycle. Either one expanded blastocyst or one expanded blastocyst plus one morula were transferred to each recipient females in 2-day culture group.

Results

Of 2,297 bovine oocytes exposed to in vitro maturation during a 30-day experiment period, 2,108 (92%) matured, 1,917 (83%) were fertilized, 1,674 (73%) cleaved, 1,048 (47%) developed to the morula stage and 317 (14%) developed to the expanded blastocyst stage. An additional 300 embryos produced during this 30-day period were cultured in TCM-199 to 16-cell, morula or early blastocyst stages on bovine cumulus cells and then frozen using a standard laboratory freezing procedure (Figure 5). These embryos were stored in liquid nitrogen at -196°C for later use.

The results of thawing, culturing and transferring the frozen IVF-derived embryos are summarized in Table 25. Of the 98 frozen embryos that were randomly selected and thawed, 58 were incubated for up to 4 h, of which 36 (62%) were determined to be
Figure 5. IVF-derived embryos produced from this study. A group of 300 embryos were frozen at 8- to 12-cell stage (a), 16-cell stage (b), morula stage (c) and early blastocyst stage (d). Ninety eight of these frozen embryos at 16-cell, morula and blastocyst stages were thawed, cultured and transferred to the recipient females. Magnifications: a, X100; b, c and d, X600.
Table 25. Results of culturing and transferring frozen-thawed *in vitro* fertilized bovine embryos

<table>
<thead>
<tr>
<th>Culture interval</th>
<th>No. of embryos thawed</th>
<th>No. of embryos transferred (%)</th>
<th>No. of recipients</th>
<th>No. of pregnancy* (%)</th>
<th>Offspring born (sex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤4 h</td>
<td>58</td>
<td>36 (62)</td>
<td>20</td>
<td>4 (20)</td>
<td>2 (1F, 1M)</td>
</tr>
<tr>
<td>≥2 days</td>
<td>40</td>
<td>12b (30)</td>
<td>10</td>
<td>5 (50)</td>
<td>3 (3M)</td>
</tr>
<tr>
<td>Total</td>
<td>98</td>
<td>48 (49)</td>
<td>30</td>
<td>9 (30)</td>
<td>5 (1F, 4M)</td>
</tr>
</tbody>
</table>

*Pregnancy diagnosed 75 days after transfer.

bTransferred at least one expanded blastocyst/recipient.
viable. Of the 40 embryos cultured for 2 days, 12 (30%) appeared viable after the culture interval, and 10 (25%) of these developed to the expanded blastocyst stage.

The 36 viable embryos cultured for up to 4 h were transferred to 20 recipients, and the 12 viable embryos that co-cultured for 2 days were transferred to 10 recipient females. Nine (30%) of the 30 recipients receiving frozen-thawed embryos were diagnosed pregnant. Only 4 of the 20 (20%) recipients received embryos cultured for up to 4 hours were diagnosed pregnant; whereas, 5 of the 10 (50%) recipients with embryos cultured for 2 days were determined to be pregnant.

Five live healthy calves were born after a 278- to 295-day gestation interval, with body weights ranging from 37.3 to 54.5 kg (Figure 6). The 4 embryos that implanted and then failed to survive, were lost after 75 days of pregnancy. One calf died of unknown causes shortly after birth, but the remaining calves gave every indication of being normal, and nursed without difficulty. These calves continued to grow and develop normally to adulthood.

The survival rates of the 40 frozen-thawed embryos that cultured for 2 days are shown in Table 26. Of the embryos that were co-cultured, 67% of the early blastocyst stage embryos and 46% of the morulae developed to expanded blastocysts; whereas, only 8.3% of the 16-cell stage embryos developed to expanded blastocyst after the co-culture interval.

Discussion

This high maturation and fertilization rates achieved demonstrated that the in vitro fertilization procedure used for bovine oocytes was efficient for the production of pre-blocked embryos. The cumulus cell co-culture system was able to provide a suitable
Figure 6. First frozen-thawed IVF-derived embryo resulting in live birth (a), and other calves and recipient cows from the same study (b). Although the breed types of the calves contributed to the range in birth weights, all calves were vigorous and developed normally during the post partum nursing period.
Table 26. Development of frozen-thawed *in vitro* fertilized bovine embryos after co-culture with cumulus cells

<table>
<thead>
<tr>
<th>Stage of thawed embryos</th>
<th>No. of embryos cultured</th>
<th>No. of expanded blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-cell</td>
<td>24</td>
<td>2 (8.3)</td>
</tr>
<tr>
<td>Morulae</td>
<td>13</td>
<td>6 (46)</td>
</tr>
<tr>
<td>Early blastocysts</td>
<td>3</td>
<td>2 (67)</td>
</tr>
<tr>
<td>Total =</td>
<td>40</td>
<td>10 (25)</td>
</tr>
</tbody>
</table>
environment for the early embryos to overcome the 8- to 16-cell *in vitro* developmental block and develop to the 16-cell and the early morula stages. However, Zhang et al. (1992c) have recently suggested that there is another decline in embryo development between the morula stage and the blastocysts stage during the *in vitro* culture of *in vitro* fertilized bovine embryos, and further study may therefore be needed to develop a better *in vitro* culture system in order to overcome this secondary *in vitro* block.

The results of culturing frozen-thawed *in vitro* fertilized bovine embryos (Table 26) clearly indicated that more later stage embryos (morulae and early blastocysts) survived freezing and culturing (>46%) than similarly frozen-thawed 16-cell stage embryos (<10%). This finding suggests that when culturing frozen-thawed, *in vitro*-produced embryos, late morula or early blastocyst stages are likely to result in a greater pregnancy rate after transfer.

The pregnancies and the birth of healthy calves after the transfer of frozen-thawed *in vitro* fertilized embryos verifies that embryos that are matured, fertilized and cultured *in vitro* are viable after freezing. One advantage may be the highly selected embryos provided by using embryo culture after thawing. In the present study, a higher pregnancy rate (50%) resulted when frozen-thawed embryos were co-cultured for 2 days and developed to the blastocyst stage before being transferred than when embryos were cultured for up to 4 hours before being transferred. This pregnancy rate is similar to that achieved by frozen-thawed bovine embryos (48.4%) derived *in vivo* at commercial transplant units (Munar et al., 1990). Long term culture increased the selection effect for embryos of transferable quality when compared with short term culture (30% vs. 62%), however, the overall efficiency (number of pregnancies per number of embryos
thawed) was also considered higher for long term cultured embryos. One possible explanation may be that transferring embryos at the blastocyst stage may produce a stronger embryonic signal to trigger the maternal recognition of pregnancy than transferring embryos at the 16-cell and morula stages.

The normal calves born during this study indicate that embryos that have been matured, fertilized and cultured \textit{in vitro} can survive freezing, and culturing and finally develop to term after being transferred to recipient cattle. This success with \textit{in vitro} fertilization has encouraged the authors to make use of the oocyte resource from slaughterhouse animals to produce a laboratory supply of bovine embryos for research purposes. Producing calves from frozen-thawed \textit{in vitro} fertilized embryos also affords opportunities to enhance the reproductive efficiency of farm animals and of rare, endangered species.
CHAPTER 9

CONCLUSION

The *in vitro* fertilization technology for cattle has developed rapidly during the past decade. Laboratories have been successful with their IVF protocols with proof of viability resulting with embryos producing live young after embryo transfer. However, the success rates of many IVF procedures are variable and the overall efficiency of current *in vitro* systems is still low. To evaluate factors affecting the successes of *in vitro* production of bovine embryos, a series of experiments were conducted in the present study.

A simple and efficient IVM/IVF/IVC procedure was developed, which produced a large number of prehatched bovine embryos for research and for embryo transfer. Oocytes that harvested from abattoir ovaries were matured in hormone-free medium, inseminated with Ca++ ionophore A23187 treated sperm and co-cultured with endogenous cumulus cells. Embryos derived from this procedure progressed rapidly through the 8- to 16-cell *in vitro* developmental block and resulted normal calves after being frozen, thawed and transferred into recipient animals. With this procedure, maturation rate of 90 to 95%, fertilization rate of 85 to 90%, cleavage rate of 65 to 75%, and morula and blastocyst development of 45 to 50% were consistently resulted after processing over 6,000 bovine oocytes in this study.

An experiment that compared three ovary holding temperature (0°C, 18°C and 25°C) verified the detrimental effects of cooling ovaries after collection on the development competence of oocytes. When ovaries were held at 0°C 3 for hours during
transportation to the embryo laboratory, the rates of oocyte maturation and fertilization were significantly reduced, and none of the oocytes cleaved from that group. When ovaries were held at 18°C for 3 hours, only 8% of oocytes developed to morula stage and none hatched, which was significantly lower than that of 25°C (50% and 20%). It appears that holding temperature of 25 to 30°C is suitable for bovine ovary transportation than lower temperatures based on the results from the present study. Therefore, in our IVF protocol, ovary transportation and subsequent handling (oocyte aspiration, evaluation and washing) are now preformed at room temperature (25-28°C).

The optimal incubation temperature and CO₂ level were found very important for the in vitro maturation and fertilization of bovine oocytes. An experiment of comparing different combinations of two temperatures (37°C vs. 39°C) and two CO₂ levels (2.5% vs. 5%) during maturation and fertilization showed that only 8% of 600 oocytes cleaved when matured and fertilized both at 37°C under 2.5% CO₂. The cleavage rate was significantly increased when oocytes were matured at 37°C, but fertilized at 39°C in 2.5% CO₂ (27.6%). A similar cleavage rate was obtained when oocytes were both matured and fertilized at 39°C in 2.5% CO₂ (24.7%). The highest cleavage rate was resulted when oocytes were matured and fertilized at 39°C with the CO₂ level raised to 5% (78.7%). These results indicate that the incubation temperature of 39°C is critical for bovine oocyte fertilization, but not for maturation. Furthermore, low CO₂ levels may have a negative effect on both maturation and fertilization of bovine oocytes.

In an experiment on cumulus cell function during IVM/IVF/IVC, cumulus cells were removed before and after maturation and after fertilization for 7, 20 and 48 hours. The cumulus-free oocytes or embryos were either cultured in medium alone or co-
cultured on cumulus cell monolayers that prepared on the day of maturation culture. Cumulus cell removal before maturation significantly reduced the rates of oocyte maturation (4 to 26% vs. 93 to 96%) fertilization (0 to 9% vs. 91 to 92%), and in vitro development at all stages evaluated. Cumulus cell removal immediately prior fertilization or 7 hours after fertilization reduced the rates of fertilization (58 to 71% vs. 91 to 92%), cleavage (40 to 54% vs. 74 to 78%), and morula plus blastocyst development (15 to 24% vs. 45%). Cumulus cells removal at 20 hours after IVF resulted in similar rates of development to that of control (cumulus cells removal at 48 hours after IVF) at all stages evaluated in this study.

The results from this study demonstrated that cumulus cells are important and necessary for oocyte maturation and acquisition of full embryonic development competence. The intact state of surrounding cumulus cells are beneficial to the oocyte and subsequent embryo development at least up to 7 hours but not at 20 hours after insemination of oocytes. The results also showed that the co-culture of denuded oocytes improved the rates of maturation and fertilization. However, this improvement did not overcome the detrimental effect caused by breakdown of the intercellular connections between cumulus cells and oocytes before and after maturation. This study verified the relationship of cumulus cells and oocytes at early developmental stages, and provide important information for in vitro fertilization and gamete micromanipulation research.

Bovine oviduct epithelial cells (BOEC) and bovine cumulus cells (BCC) are most commonly used helper cells for co-culture of IVF-derived bovine embryos. In the experiment of evaluating these two co-culture systems, results showed that cumulus cell co-culture consistently supported more 2- to 8-cell stage embryos developing to morula
and blastocyst stages. This was also the case in either simple defined serum-free medium (CZB) or in complex serum-containing medium (TCM-199). When BOEC and BCC was used in combination for embryo co-culture, the development rates were similar to that of BCC co-culture at all stages tested. This result further established that BCC co-culture is stable in supporting early bovine embryo development in vitro. However, the effect of BOEC on early development may depend to some degree on the stage of estrous cycle of the cow from which the cells are harvested.

When two culture media were compared in this study (CZB vs. TCM-199), the results showed that more early stage embryos developed to morulae in serum-free CZB than in serum-containing TCM-199, but the embryo development in CZB medium was slowed down thereafter and significantly fewer embryos hatched in CZB than in TCM-199. It appears that serum inhibited embryo cleavage at early development stages but stimulated the blastocyst formation, expansion and hatching. Findings from this study suggest that a culture system that starts with serum-free medium and then systematically increases the serum concentration to fit the embryo developmental stage may be more suitable for in vitro production IVF-derived bovine embryos.

Although there are evidences that growth factors involved in pre-hatched embryo development, how this factors regulate this early developmental process remains to be determined. In the present study, eight growth factors/mitogens (insulin, GH, EGF, FGF, IGF-1, PDGF, PGF_2α and AA) were investigated by adding them to the culture medium and monitoring the embryo development in vitro. Among these growth factors, arachidonic acid was found to have a marked stimulating effect on IVF-derived bovine embryos. Arachidonic acid promoted early stage embryos developed to morula,
blastocyst and hatching stages, and this effect was significantly greater than the other treatments both in co-culture or medium alone culture. The optimal arachidonic acid concentration was found to be 50 ng/ml of culture medium, and high concentration (500 ng/ml) actually inhibited blastocyst formation. This was the first time that direct evidence of arachidonic acid stimulating bovine embryo development has been reported. Further study is necessary to reveal the mechanism of this response.

Beside arachidonic acid, insulin was found to be able to stimulate cumulus cell expansion during maturation and increase the rate of in vitro fertilization for bovine oocytes. Insulin also stimulated the embryonic cell proliferation by increasing the number of cells/embryo at the morula, blastocyst and hatched blastocyst stages when compared with the control group. This finding suggests that exogenous insulin participated in the metabolism of incubated oocytes and further indicated that insulin is involved in early embryo development.

Other growth factors evaluated in this study had no detectable effect on in vitro embryo development when compared with the control treatment. This may be explained by the presence of a cooperative interaction among cultured bovine embryos. It has been found that embryos can produce growth factors as well as express receptors for those autocrine/paracrine growth factors. Since the embryos were group cultured in these experiments, the effect of exogenous growth factors could have been masked by embryo origin growth factors that participate in embryo development. Therefore, a more controlled culture system is necessary (e.g. continuous flow system) to study the function of growth factor during early embryo development. The other possible explanations are the exogenous growth factors may be not needed for prehatched bovine embryo
development, or the concentrations of exogenous growth factors used in this study were too low in those experiments to affect the embryo development.

Research and commercial use of prehatched embryos ultimately requires embryo cryopreservation. Although bovine embryos can be produced in the laboratory, it has been found that \textit{in vitro} produced embryos are much more sensitive to freezing than \textit{in vivo} produced embryos. To test the viability of embryos produced from the present study, 300 IVF-derived embryos were frozen at 16-cell, morula or early blastocyst stages. Some of this frozen embryos were thawed 10 to 12 month later and either transferred after 4 hours or co-cultured for 2 days to allow embryos develop to the expanded blastocyst stage and then transferred into recipient animals.

The survival and development of frozen-thawed IVF-derived embryos were much greater when embryos were frozen at relatively later developmental stages than at an early stage. The expanded blastocyst development rate was 8.3\% for 16-cell stage embryos, 46\% for morulae and 67\% for early blastocysts. Higher pregnancy rate (50\%) resulted when frozen-thawed embryos were co-cultured and developed to expanded blastocysts before transfer compared with those of cultured for only a few hours and transferred (20\%). Extended \textit{in vitro} culture increased the selection effect for embryos of transferable quality when compared with a shorter term culture (30\% vs. 62\%). Furthermore, the overall efficiency (number of pregnancies/number of thawed embryos) was also greater for embryos cultured \textit{-} 2 days. Nine pregnancies were established from embryo transfer of 30 recipient cows and 5 calves were born after a normal gestation period. These results demonstrate that \textit{in vitro}-produced bovine embryos are viable in terms of producing live young. These results also showed that the survival of the frozen-
thawed IVF-derived embryos that developed to term after embryo transfer are likely to be obtained from blastocyst-stage embryos. These calves were the first frozen-thawed, IVF-derived offsprings in the USA.

The methodology of in vitro production of bovine preimplantation embryos has been established and now being used for both research and commercial purposes. However, the underlying mechanism of gamete interaction and early mammalian development is far from fully understood. Further studies on the requirement and regulation of these early development are needed to better understand the early development, as well as finding of an optimal in vitro system for laboratory embryo production. The ability of producing viable prehatched embryos will, in turn, provide a continuous laboratory supply. Thus, stimulating the basic studies especially molecular biology research on early embryos that is now difficult without large number of bovine embryos. The progresses in resent years are very encouraging. The successes on in vitro fertilization and embryo production will also afford opportunities to enhance the reproductive efficiency of farm animals and of rare, endangered species.
LITERATURE CITED


APPENDIX A

BRACKETT-OLIPHANT MEDIUM

<table>
<thead>
<tr>
<th>Item</th>
<th>mM</th>
<th>100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-O Stock Solution (10X)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>112.00</td>
<td>6.550 g</td>
</tr>
<tr>
<td>KCl</td>
<td>4.02</td>
<td>0.300 g</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>0.83</td>
<td>0.115 g</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.52</td>
<td>0.106 g</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>2.25</td>
<td>0.331 g</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>13.90</td>
<td>2.518 g</td>
</tr>
<tr>
<td>Penicillin-G</td>
<td>—</td>
<td>100 000 IU</td>
</tr>
<tr>
<td>Streptomycin SO₄</td>
<td>—</td>
<td>100 000 μg</td>
</tr>
<tr>
<td>0.5% Phenol red</td>
<td>—</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>Distilled Milli-Q water raise to 100 ml total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B-O Medium (100 ml)

<table>
<thead>
<tr>
<th>Item</th>
<th>mM</th>
<th>100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>37.00</td>
<td>0.3104 g</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>1.25</td>
<td>0.0138 g</td>
</tr>
<tr>
<td>B-O Stock Solution (10X)</td>
<td>—</td>
<td>10 ml</td>
</tr>
<tr>
<td>Distilled Milli-Q water raise to 100 ml total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To prepare B-O Stock Solution:

1. In 60 ml of distilled Milli-Q water, dissolve NaCl, KCl, NaH₂PO₄·H₂O, D-glucose, penicillin-G, potassium, and streptomycin sulfate.

2. In 10 ml of Milli-Q water, dissolve CaCl₂·2H₂O and MgCl₂·6H₂O. Then slowly add solution from step 2 to the solution prepared in step 1.

3. Add 1.6 ml phenol red (optional).

4. Add Milli-Q water to make a final volume of 100 ml and then filter through a 0.2-μm filter, and aliquot into sterile containers.

5. Store at 4°C for up to 3 mo.

To prepare B-O Medium:

1. In 80 ml of Milli-Q water dissolve NaHCO₃ and sodium pyruvate.

2. Add 10 ml of B-O Stock Solution and then q.s. with Milli-Q water to make a final volume of 100 ml.
3. Adjust pH to between 7.6 and 7.8 and osmolarity to 270 and 290 mOsm.

4. Filter through a 0.2-μm filter and aliquot into sterile vessels.

5. Store at 4°C for up to 1 mo.
APPENDIX B

PREPARATION OF Ca++ IONOPHORE A23187
FOR SPERM CAPACITATION

Ca++ ionophore A23187 solution (1 mM)

1. In 1.9 ml of dimethylsulfoxide dissolve 1 mg Ca++ ionophore A23187.
2. Aliquot 50 μl of the ionophore solution into sterile containers (microvials).
3. Store the aliquots in the dark at -20°C for up to 6 mo.
APPENDIX C

COMPOSITION OF CZB MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/ml</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>4767</td>
<td>81.62</td>
</tr>
<tr>
<td>KCl</td>
<td>360</td>
<td>4.83</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>161</td>
<td>1.18</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>291</td>
<td>1.18</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2110</td>
<td>25.12</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>250</td>
<td>1.70</td>
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<tr>
<td>Na pyruvate</td>
<td>29.7</td>
<td>0.27</td>
</tr>
<tr>
<td>Na lactate</td>
<td>3609</td>
<td>31.30</td>
</tr>
<tr>
<td>EDTA (disosium salt)</td>
<td>41.8</td>
<td>0.11</td>
</tr>
<tr>
<td>Glutamine</td>
<td>146</td>
<td>1.00</td>
</tr>
<tr>
<td>BSA (mg/ml)</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Na penicillin-G (IU/ml)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Streptomycin (mg/ml)</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

(Chatot et al., 1989)
VITA

Li Zhang, the daughter of Jingshan Zhang and Qingmei Liang, was born in Beijing, China on January 17, 1956. She received her elementary and secondary education at Beijing Tielu No. 5 Elementary School and Beijing Tielu No. 3 Middle School in Beijing, China. After passing the national examination to enter the college in August, 1978, she enrolled in Beijing Normal University where she received the degree of Bachelor of Science with a major in biology in August, 1982. For the following year, she was employed as an instructor in Beijing Fengtai Agricultural Technology School.

In August, 1983 she entered the Graduate School at Chinese Academy of Agricultural Sciences. She was awarded the degree of Master of Science in August, 1986 with a major in Animal Reproductive Physiology, and at the same time she accepted a research associate position in the Institute of Animal Science, Chinese Academy of Agricultural Sciences. In April, 1989 she become a visiting scientist in the Department of Animal Science, Cornell University. The same year in September, she moved to Louisiana State University and worked in Animal Science Department as a research associate.

In August of 1990, she started her doctor training program in Reproductive Physiology under the guidance of Dr. Robert A. Godke in the Department of Animal Science at Louisiana State University. She was awarded the degree of Doctor of Philosophy in December of 1995.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Li Zhang

Major Field: Animal Science

Title of Dissertation: In Vitro Maturation, In Vitro Fertilization and Development of Bovine Oocytes

Approved:

[Signatures]

Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: October 19, 1995