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Effects Of Culture Methods And Simulated Microgravity Conditions On Development Of Bovine Embryos Produced In Vitro

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EFFECTS OF CULTURE METHODS AND SIMULATED MICROGRAVITY CONDITIONS ON DEVELOPMENT OF BOVINE EMBRYOS PRODUCED *IN VITRO*

By

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EFFECTS OF CULTURE METHODS AND SIMULATED MICROGRAVITY

CONDITIONS ON DEVELOPMENT OF BOVINE EMBRYOS

PRODUCED *IN VITRO*

By

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The objectives of this study were (1) to determine the optimum *in vitro* culture conditions for *in vitro* fertilized bovine embryos among culture methods and (2) to investigate whether bovine fertilization and embryo development would occur in simulated microgravity conditions *in vitro*. In the first part of this study, the result showed that the microdrop method was the optimum culture method among groups; however, FBS supplementation did not significantly affect the bovine preimplantation embryo development *in vitro*. In the second part of this study, the result showed that bovine *in vitro* fertilization did not occur in simulated microgravity conditions. Moreover, none of the presumptive zygotes and 2-8 cell stage embryos were able to develop to further stages, while embryos cultured in standard microdrop method culture conditions developed normally.

Key words: bovine, *in vitro* fertilization, *in vitro* culture, embryo development, culture methods, simulated microgravity

DEDICATION

I would like to dedicate this thesis to my grandfather, Lee Won, for your support, your encouragement, your advice, and your prayers.

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CHAPTER I

INTRODUCTION

Bovine *in vitro* embryo production (IVP) has been developed and widely used as a research tool of basic mechanisms of fertilization and embryogenesis as well as an application for animal biotechnologies. *In vitro* production of bovine embryo is composed of essentially three biological steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). In particular, the procedures of IVC have an affect on the quality and viability of *in vitro* produced embryos since embryos are cultured up to 8 days (Galli et al., 2003). Various components of the *in vitro* culture, such as the quality of serum and oil, culture medium, embryo density, and protein supplement influence the developmental competence of mammalian embryos (Bavister, 1995). For that reason, the developmental competence of embryos can be varied by different culture methods, and their efficiencies also differ from one laboratory to another. Therefore, it is important to examine these components and determine optimum culture methods affecting the development of *in vitro* produced embryos.

There have been many biological experiments conducted during space flights and in simulated microgravity conditions to investigate the effects of long-term space travel and to determine the influence of gravity on development of living organism on the Earth

(Committee on Space Biology and Medicine, 1998; White and Averner, 2001). Studies conducted during and after spaceflight have reported that significant physiological changes in astronauts and experimental animals (Planel, 2004), as well as mammalian cells exposed to the microgravity conditions for only a few days show changes in function and structure (Hughes-Fulford, 1991). Experiments have been performed to determine the possibilities of normal fertilization and embryo development under microgravity conditions. Studies in fish and amphibians have shown that their fertilization and embryo development could occur in space and simulated microgravity conditions (Crawford-Young, 2006). In mammals, fertilization and embryo development have not yet been studied with a variety of species either during space flight or simulated microgravity conditions. Only studies in the rodent have been conducted, and their results have not been consistent. Thus, more studies need to be completed concerning fertilization and preimplantation embryo development in various mammalian species to further examine the influence of microgravity on developmental processes.

The goal of this thesis is to evaluate the components that affect bovine *in vitro* culture and determine the optimum *in vitro* culture method for bovine embryos, and to determine whether bovine *in vitro* fertilization and preimplantation embryo would occur in simulated microgravity conditions.

CHAPTER II

LITERATURE REVIEW

Oocyte and Embryo

Oocyte Origin

The development of mammalian oocytes and sperm begins with the formation of primordial germ cells (PGCs) in embryos. The PGCs are formed from the extra-embryonic ectoderm, induced by neighboring epiblast cells (Gilbert, 2003). In the mouse, these PGCs can be first detected in the posterior primitive streak around 7 days post-coitum (pc) because of their high level of tissue nonspecific enzyme alkaline phosphatase activity (Ginsburg et al., 1990). In the bovine, alkaline phosphatase - positive PGCs are observed in the 18 day old embryo (Wrobel and Suss, 1998). Then, PGCs proliferate and migrate from the yolk sac epithelium through the gut mesentery to the genital ridges of the early embryo around 10 days pc in the mouse (Gilbert, 2003). Once PGCs complete the colonization in the genital ridges, they differentiate either into oogonia or spermatogonia depending on the available sex chromosomes (Van den Hurk and Zhao, 2005).

In the female, these oogonia expand their population through a fixed and species-specific number of mitotic divisions (De Felici et al., 2005). In the mouse during this time,

the number of oogonia increases to 1000 at 10.5 days pc and, finally, to a maximum number of 20,000-25,000 at 14.5 days pc when they begin meiosis (Godin et al., 1990). Expansion of the population of oogonia lasts longer in the bovine and human. The total number of oogonia in the bovine rises dramatically from 16,000 on 50 days pc, to 2,739,000 on 110 days pc (Barker and Hunter, 1978). In humans, it appears to increase from some 600,000 at 2 months pc reaching a peak of 6,800,000 at 5 months pc (Barker, 1963). However, there is also germ cell loss by 'programmed cell death' or apoptosis that can occur at the same time as proliferation. The number of germ cells starts to decrease dramatically to 2,000,000 by the time of birth in the human (Barker, 1963). In the mouse, by the time of transition from mitosis to meiosis, approximately 70% of germ cells are lost by the time of birth (Fulton et al., 2005).

After PGCs colonization at the gonad, oogonia are likely to aggregate and develop in clusters or nests, and then they form cysts (Gomperts et al., 1994). These germ cell clusters are connected to each other by intracellular cytoplasmic bridges, which allow continuous communication between them and coordinate synchronous mitotic divisions (Pepling, 2006). In parallel to the germ cell proliferation, somatic cells, i.e. granulosa and theca cells, also proliferate and lead to the rapid growth of the genital ridges. In the developing ovary, the oogonia interact with somatic cells, and both of them progressively organize epithelial structures, called ovigerous cords that are developed later into a follicular unit comprised of a single oocyte (Guigon and Magre, 2006).

Oocyte and Follicular Development

Oogonia undergo the two cell divisions of meiosis to reduce their diploid chromosome numbers to haploid states and generate a genetic recombination of maternally and paternally derived DNA information. When oogonia begin the first meiotic division, the germ cell cysts are disassembled. After that, oogonia differentiate into primary oocytes. These oocytes progress through leptotene, zygotene and pachytene stages, and then they become arrested in diplotene or dictyate stage of prophase I (germinal vesicle stage oocytes; GV) (Betteridge et al., 1989; Gordon, 2003). In the human, meiosis starts at the end of the first trimester of pregnancy. The mid-gestational human fetal ovary contains both oogonia and diplotene-stage oocytes (Picton et al., 1998). In the bovine, diplotene-stage oocytes increase between 170 days of gestation and the time of birth (Barker and Hunter, 1978). Meiosis does not resume until puberty.

Follicular development starts from the central part of ovary immediately after the first oocyte reaches the diplotene stage (Pepling, 2006). Folliculogenesis is the process of primordial follicle recruitment, follicular growth, and selection and maturation of the preovulatory follicle. The follicles play a role in maintaining the oocyte in a controlled environment and protecting them from harmful substances. Also, these follicles are responsible for the maturation of the oocytes by producing steroid and peptide sex hormones and other compounds (Gordon, 2003).

The first step of follicular development is that follicular (pre-granulosa) cells proliferate and surround oocytes with a single layer of flattened somatic cells. This unit is referred to as a primordial follicle (Van den Hurk et al., 2000). During this period, about

20-30% of oocytes are enclosed and survive within primordial follicles. The rest of the oocytes are rapidly degenerated by apoptosis. After follicular stages, oocyte death occurs as a result of follicle atresia (De Felici et al., 2005). Pre-granulosa cells of a primordial follicle develop into a layer of cuboidal granulosa cells around the primary oocyte, forming the primary follicle.

The cuboidal granulosa cells multiply and form several layers, at which time follicles are then termed preantral follicles. During this stage, the oocytes increase in volume without cell divisions, and several stromal cells differentiate into theca cells forming a layer that surrounds the follicle. The zona pellucida is visible and associated with the formation of gap junctions with the oolemma between the oocyte and the inner layer of the granulosa cell. The granulosa cells nourish and regulate further oocyte development via these gap junctions. The function of gap junctions is characterized by helping the communication between the oocyte and the granulosa cells with blood vessels (Betteridge et al., 1989; Fair et al., 1997; Telfer, 1996). Oocytes in preantral follicles start to synthesize and store transcripts and proteins essential for maturation, fertilization, and preimplantation embryo development; yet, these oocytes are not able to resume meiosis.

When follicles have reached the antral stage of development, oocytes of most species have acquired the ability to resume and complete meiosis (Telfer, 1996). Antral follicles are characterized by the antrum formation, which occurs with accumulation of fluid between multilayered granulosa cells. Antrum fluid is a unique body fluid containing a large number of agents derived from blood and other local secretions (Van den Hurk and Zhao, 2005). From antral stage to preovulatory stage, follicle development

is under the influence of pituitary follicle stimulating hormone (FSH) (Loutradis et al., 2006). The oocytes and follicles continue to grow and results in either ovulation of a mature oocyte, or degeneration (atresia) of the follicle and its oocyte.

Under the influence of FSH, an antral follicle reaches the preovulatory stage among the pool of early antral follicles. The selected follicles in humans and bovine are called dominant follicles. In the dominant follicle, an oocyte is surrounded by closely associated granulose cells, or cumulus cells, forming a compact cumulus cell-oocyte complex (COC) (Van den Hurk et al., 2000). The remaining large follicles undergo atresia.

Cell Cycle Overview

In growing cells, the cell cycle is composed of four major phases, G1 (Gap 1), S phase (DNA synthesis), G2 (Gap 2), and M phase (Mitosis). The interphase in which the nucleus is visible and intact under the light microscope encompasses G1, S phase, and G2. During G 1 phase, cells grow and double their mass of proteins and organelles in the cytoplasm. The DNA synthesis occurs during the S phase, and then these cells, with duplicated chromosomes which are called sister chromatids, pass into G2 phase where cell growth is resumed, and all the proteins necessary for cell division are produced. Subsequently, the cells enter M phase, which is divided into prophase, prometaphase, metaphase, anaphase, and telophase. First, the chromosome condensation occurs in order to undergo their cell divisions without losing any genetic information, and the spindle starts to form outside of nucleus. This stage of mitosis is called prophase. Next, during

the prometaphase, the nuclear envelope breaks down, and microtubules of the mitotic spindle attach to each sister chromatid. The chromosomes become aligned at the equator of the mitotic spindle, forming the metaphase plate. This state is called metaphase. Then, the paired sister chromatids separate and move to opposite poles of the mitotic spindle, segregating one of the two sister chromatids to each daughter cell during anaphase. Once the chromosomes arrive at the opposite pole of the cell, new membranes are formed around the daughter nuclei and chromosomes decondense during telophase. Cytokinesis, which is the physical division of the cytoplasm, begins during this stage. Finally, the cells divide into two daughter cells. A standard mammalian cell cycle is about 18 to 24 hours. Interphase might occupy about 23 hours, while M phase requires less than 1 hour (Alberts et al., 2002a; Kiessling and Anderson, 2003b).

Oocyte Maturation

In the dominant follicle, the oocyte remains arrested at the diplotene stage of the meiotic prophase I. *In vivo*, oocyte maturation and expansion of cumulus cells are initiated by a preovulatory Luteinizing Hormone (LH) surge and only occurs in fully grown competent oocytes from dominant follicles (Salha, 1998). There are several changes in the follicular cells responding to LH surge. First, the steroid production is shifted by cumulus cells from predominately estrogen to progesterone synthesis, and hyaluronic acid is produced by cumulus cells. This leads to the mucification or expansion of the cumulus cells with the accompanying termination of gap junctional contact between the granulosa cell and the oocytes in the follicles. The loss of intercellular

communication between the oocyte and its surrounding granulosa cells may serve as a trigger for the resumption of meiosis in mature oocytes *in vivo* since when immature cumulus cell-oocyte complexes (COCs) are removed from their follicular environment and cultured in a simple maturation medium, meiosis spontaneously resumes. Several studies have demonstrated that unidentified factors secreted by granulosa cells maintain bovine oocytes in the germinal vesicle stage (GV) because co-culture of oocytes and granulosa cells prevents them from meiotic resumption *in vitro* (Edwards, 1965; Richard and Sirard, 1996). These changes in the follicular cells occur simultaneously with nuclear and cytoplasmic maturation of the oocyte.

The nuclear maturation involves the change of the chromatin status from the diplotene stage of prophase I (GV stage) to metaphase of the second meiotic division (MII) (see Appendix B). It is mediated by the production of active Maturation Promoting Factor (MPF), a protein complex composed of cyclin B1 and $P32^{cdc2}$, in ooplasm (Van den Hurk and Zhao, 2005). The LH surge induces a germinal vesicle breakdown (GVBD), which is directed by MPF. GVBD involves a disappearance of the nucleolus, dissociation of the nuclear membrane, condensation of the chromatin, and reorganization of the microtubular complex to form a functional spindle apparatus. The maturation of the oocyte progresses to the first metaphase (MI), and the replicated homologous chromosomes segregate into two daughter nuclei at anaphase I (AI). At telophase I (TI), the cytoplasm divides asymmetrically, which results in the formation of large oocyte and the small polar body each containing a complete set of chromosomes. The second meiotic division commences without formation of any interphase nucleus, and then it is finally

rearrested in meiosis at the metaphase II (MII) stage until fertilization. An activation stimulus provided by sperm penetration triggers the completion of the second meiotic cycle and initiates embryonic development (Alberts et al., 2002b; Kiessling and Anderson, 2003a; Sirard et al., 2006). Nuclear maturation lasts about 24 hours in the cow and sheep, about 44 hours in the pig, and about 36 hours in the horse (Edwards, 1965).

The quality of the oocyte depends on cytoplasmic maturation. Although nuclear maturation has progressed normally, many of these "mature" oocytes may be developmentally incompetent due to incomplete or abnormal maturation of the cytoplasm. Oocytes with insufficient cytoplasmic maturation are unable to be fertilized and develop further. The cytoplasmic maturation is associated with all the changes in distribution and organization of organelles from the GV to the MII stage. It includes migrations of mitochondria to the inner cytoplasm and cortical granules to the periphery of the ooplasm. In addition, the mechanisms for sperm head penetration, decondensation of chromatin, and the polyspermy block are all progressively acquired during the final days of maturation before ovulation. Another event that takes place during the cytoplasmic maturation is the accumulation of RNAs and proteins. Most of the RNA present in oocytes is synthesized during folliculogenesis. However, RNA synthesis decreases before GVBD, so that embryo development depends on mRNA and proteins already stored in the cytoplasm during the period before ovulation (Hyttel et al., 1989; Loutradis et al., 2006; Sirard et al., 2006; Van den Hurk and Zhao, 2005).

Oocyte and Sperm Fertilization

The process of fertilization is a chain of events that involve receptor-ligand interactions, membrane fusions, signaling cascades, specific proteolysis, nuclear transformations and other events (Wassarman, 1999). It begins when spermatozoa approach a mature oocyte within the female oviduct. The haploid spermatozoa are produced within the seminiferous tubules of the testis. Then, these spermatozoa are transported through the epididymis and undergo additional biochemical and functional modification. They are then stored within the cauda epididymis, where they are actively motile yet unable to fertilize the oocyte (Evans and Florman, 2002; Shur et al., 2004).

After spermatozoa enter into the female reproductive tract, they undergo a maturation process called capacitation before reaching the mature oocyte in the oviduct. The length of time needed for capacitation varies from species to species but usually requires several hours. Capacitation is associated with removal of adherent seminal plasma proteins and reorganization of plasma membrane lipids and proteins. It also involves an influx of extracellular calcium, increase of cyclic AMP, and decrease in intracellular pH. These changes lead to displaying hyperactive motility patterns of spermatozoa and destabilizing the sperm's membrane for binding and interacting with the oocyte (Senger, 1999; Shur et al., 2004; Yanagimachi, 1981). Moreover, throughout capacitation spermatozoa gain the ability to respond to a chemotatic signal from the ovulated oocyte (Wassarman, 1999). In many species, including human, capacitation can be completed by incubation within a variety of cell culture media, which contain Ca^{2+}, BSA, and biocarbonate (Storey, 1995). Hyperactive motility facilitates sperm-oocyte

contact. Only capacitated spermatozoa are able to recognize the ovulated oocyte and bind its zona pellucida.

Spermatozoa are known to contain several proteins on the membrane surface overlying the acrosome. These proteins bind to species-specific receptors on the zona pellucida of the oocyte. The mammalian oocyte zona pellucida is composed of three glycoproteins, zona protein 1, 2, and 3 (ZP1, ZP2, and ZP3) that are synthesized and secreted by the oocyte. ZP1 and ZP2 are structural proteins maintaining the structural integrity of the zona pellucida. ZP3 is the major receptor for the protein found on the spermatozoal membrane (Dean, 1992; Senger, 1999; Wassarman, 1999). It has been proposed that binding the protein on sperm to ZP3 induces a signal transduction across the sperm membrane, and this process initiates the acrosome reaction. The acrosome is a membrane-bound organelle interiorly located in the head of spermatozoa, which is packed with a variety of hydrolyzing enzymes. The acrosome reaction allows the spermatozoon to penetrate the zona pellucida. The acrosomal reaction starts when the sperm plasma membrane fuses with the outer acrosomal membrane leading to leakage of hydrolyzing enzyme from the sperm's head. This enzyme hydrolyzes zona pellucida proteins so that the motile spermatozoon can easily pass through the zona pellucida. As the acrosome reaction progresses, more and more of the plasma membrane and acrosomal contents are lost (Senger, 1999; Storey, 1995; Yanagimachi, 1981, 1989).

Once a spermatozoon penetrates the zona pellucida, it adheres to and fuses with the plasma membrane of the oocyte. Then, the head of the fertilizing spermatozoon is incorporated into the oocyte cytoplasm. After membrane fusion, the oocyte undergoes a

number of metabolic and physical changes that are called oocyte activation. The oocyte activation involves an increase in the intracellular concentration of calcium, which induces the resumption of second meiosis and the cortical reaction. The cortical reaction refers to an exocytosis of cortical granules seen shortly after membrane fusion between the oocyte and spermatozoon. Cortical granules contain several proteases and glycosidases, which are released into the perivitelline space. Exocytosis of the cortical granules modifies the properties of the zona pellucida, which is known as the zona reaction. The zona pellucida hardens, and sperm receptors in the zona pellucida are destroyed in order to prevent the entry of additional sperm, which is called polyspermy (Senger, 1999; Shur et al., 2004; Sun, 2003).

Following entry of the fertilizing spermatozoon within the oocyte cytoplasm, the nuclear envelope of the sperm disperses, and the sperm nucleus decondenses from its tightly packed state. Simultaneously, the oocyte completes its second meiosis with the extrusion of the second polar body and formation of a nuclear envelope around the decondensing chromosome. Then, chromatins from both the sperm and oocyte form pronuclei. Each pronucleus contains a haploid genome. The male and female pronuclei migrate together and are fused in the center of the oocyte. The two genomes condense into chromosomes, thereby reconstituting a diploid organism. This final process of fertilization is called syngamy. The zygote enters the first stages of embryogenesis (Alberts et al., 2002b; Kiessling and Anderson, 2003a).

Functions of Cytoskeleton in Mammalian Oocyte Maturation and Fertilization

The cytoskeleton is an internal skeleton in eukaryotic cells. It comprises three components: microfilaments, microtubules and intermediate filaments that influence fundamental cellular processes including mitosis, and cytokinesis within cells (Scholey et al., 2003). Additionally, the cytoskeleton establishes cell and tissue shape and facilitates cell motility and movement of organelles and proteins within the cytoplasm (Pollard, 2003). Microtubules and microfilaments are major cytoskeletal components in mammalian oocytes and embryos, and it is also known to regulate the chromosomal and cytoplasmic dynamics during their maturation and fertilization (Gallicano, 2001; Sun and Schatten, 2006). Many studies have shown that addition of drugs that inhibit cytoskeleton polymerization or depolymerization during culture can result in failure of completing all of the developmental process during mammalian oocyte maturation and fertilization. Studies have found that microtubules play a role in chromatin reconstruction, and microfilaments are involved in chromosomal movement to the peripheral position after GVBD and polar body extrusion during the nuclear maturation in human (Kim et al., 1998), bovine (Kim et al., 2000), and porcine (Sun et al., 2001a) oocytes. In addition, microtubules and microfilaments mediate cytoplamic maturation in mammalian oocytes. Studies have shown that cortical granule migration in porcine oocytes was mediated by microfilaments (Sun et al., 2001a), while the translocation of mitochondria to the inner cytoplasm of oocyte was controlled by microtubules (Sun et al., 2001b).

In most mammals, it is known that sperm introduce a centrosome into the oocyte during fertilization. A centrosome is also called the microtubule organizing center

(MTOC), which nucleates microtubule structure and forms sperm aster (Navara et al., 1995). Studies have found that microtubules of sperm aster facilitate the migration of the male and female pronuclei from cortex to close apposition at the center of the oocyte and formation of the first mitotic spindle in the porcine (Kim et al., 1997; Sun et al., 2001a), bovine (Navara et al., 1994), and human (Nakamura et al., 2002). In the mouse, microtubules are also involved in pronuclei migration and mitotic spindle formation; however, MTOC is originated from oocyte rather than sperm unlike in other mammalian species (Schatten et al., 1985). Microfilaments also play an essential role in mammalian fertilization. Sun and Schetten (2006) demonstrated in their review that microfilaments mediate extrusion of sperm incorporation, second polar body, and cleavage ring formation. Additionally, Sun et al. (2001a) and Kim et al. (1997) have showed that syngamy and cell division was inhibited in porcine oocytes cultured in medium containing cytochalasin B, a drug that inhibits the microfilament assembly (Kim et al., 1997). Taken together, these studies suggest that interactions between microtubules and microfilaments are required for the mammalian oocyte maturation, fertilization, and subsequent cell division.

Preimplantation Cleavage

The zygote undergoes a series of cell divisions, progressing through 2-cell, 4-cell, 8-cell and 16-cell stages. These cells in cleavage stage embryos are known as blastomeres. During cleavage stages, there is no cell growth before going through mitosis because while the number of cells increases, the total volume of cytoplasm remains relatively

constant. Without a growth phase, progression through the cell cycle is relatively fast. During the first few cleavage divisions, each mitosis is followed immediately by DNA synthesis in the two daughter cells with short G1 and G2 phases unlike somatic cell division. Early cleavage divisions occur synchronously, so blastomeres in a 2 cell stage embryo undergo mitosis and cytokinesis almost at the same time. For this reason, 2-, 4-, and 8- cell stages are much more often observed than 3-, 5-, and 7- cell stages (McLaren, 1972).

In all species, the development of early cleavage divisions is dependent on maternal RNAs and proteins stored in oocyte cytoplasm. Oogenetic RNAs and proteins start to be depleted after several cleavage divisions, and they are gradually replaced by new embryonic transcripts. This transition from maternal to embryonic control is called embryonic genome activation (EGA) or zygotic gene activation (ZGA) (De Sousa et al., 1998a). In the bovine, a major portion of embryonic genome transcription is initiated at the fourth cell cycle (8- to 16-cell stages). De Sousa et al. (1998b) observed that bovine embryos cultured from the zygote to 4-cell stage in the presence of a transcriptional inhibitor (α- amanitin) progressed to the 4- to 8-cell stage but none of them develop to the 16-cell stage (De Sousa et al., 1998b). In this study, researchers also transiently detected mRNA for eukaryotic translation initiation factors at 8-cell stage of bovine embryos. This result indicates that new embryonic transcription is occurring during the fourth (8-cell) cell cycle. Moreover, changes in bovine embryonic protein synthesis occur from the fourth to the fifth cell cycle as well. Similar protein patterns of bovine embryos from *in vivo* and *in vitro* at these stages have proven that both types of embryos undergo EGA at

similar times (Barnes and Eyestone, 1990; Schultz, 2002). The time of this EGA varies between species. EGA occurs in the mouse at the 2-cell stage, in human and porcine embryos at the 4- to 8-cell stages (De Sousa et al., 1998a).

Studies have reported the duration of cell cycles of bovine embryos using the cinematographic analysis (Grisart et al., 1994; Holm et al., 1998; Van Langendonckt et al., 1997). The duration of the first, second, third, and fourth cell cycles of *in vitro* derived bovine embryos have been reported to be 34, 9 to11, 11 and 48 to 51 hours, respectively. The duration of cleavage of *in vivo* and *in vitro* derived bovine embryos was similar up to the 8-cell stage, but the development of the *in vitro* derived bovine embryos was delayed during the fourth cell cycle when compared with embryos produced *in vivo*. However, the duration of bovine embryos developed *in vivo* is 32, 13, 14 and 24 hours for the first to fourth cell cycles respectively (Grisart et al., 1994; Holm et al., 1998). The lag-phase of bovine *in vitro* derived embryos is occurred at the fourth cell cycle that is about the stage of initiation of EGA. Eyestone and First (1991) reported that similar patterns have been observed in the sheep and mouse embryos. They suggested that the time of EGA might have been associated with an increase in length of the cell cycle, which may allow for embryonic transcription to occur. The majority of bovine embryos that do not reach the blastocyst stage are usually arrested at 8-cell stage *in vitro*. Researchers speculate that the combination of suboptimal *in vitro* culture condition and the quality of oocytes could influence the lag-phase between the 8- to 16-cell stages *in vitro* (Eyestone and First, 1986; First and Barnes, 1989; Grisart et al., 1994; Sirard et al., 2006).

Several *in vitro* culture studies have shown that zygotes cleaving earliest after IVF tend to have a great chance of developing into blastocysts and hatching embryos than later cleaving zygotes. It is clearly shown that timing of early first cleavage division can be considered as a useful tool for prediction of blastocyst formation and hatching capacity (Fair et al., 2004; Holm et al., 2002; Van Langendonckt et al., 1997). The faster the embryos cleaved the more chance (up to 70%) they had of becoming morulae-blastocysts by day 8 post insemination (pi). More slowly dividing embryos develop at a very low rate and reach only the 9-16 cell stage or even earlier stages (Grisart et al., 1994).

Blastocyst Formation and Hatching

The blastocyst formation is the most critical event of embryo preimplantation development in all mammalian species. It is required for implantation and its further development of embryos. In addition, it is a major determinant of embryo quality *in vitro* prior to embryo transfer. After the fourth and fifth cell division cycles, depending on species, the embryos become morula and start to form tight junctions. It is characterized by increased cell to cell contact between blastomeres. The formation of tight junctions continues until the individual blastomere outlines disappear. This change in shape of the embryo is called compaction. Compaction occurs around the 32-cell stage *in vivo* and *in vitro* for bovine embryos (Gordon, 2003; Watson et al., 1999).

As tight junctions develop, trophectoderm is differentiated at the outer cells of the morula, and it sets a barrier between the outer embryonic blastomeres. Also,

trophectoderm begins to express a variety of membrane transport molecules, including Na/K ATPase, which is the Na^+ and K^+ transporter found in all cell membranes responsible for the transport of Na^+ out of and K^+ into the cell against concentration gradients. This enzyme establishes ion concentration gradients across the trophectoderm and facilitates the osmotic movement of water into the extracellular space to form a fluid-filled cavity of the blastocyst. As the blastocyst continues to accumulate fluid in the cavity, it expands to form an expanded blastocyst that varies distinctly in size between mammalian species. A blastocyst is composed of the outer epithelial trophectoderm and the undifferentiated inner cell mass. Trophectoderm develops into the extraembryonic membranes, while the inner cell mass gives rise to the fetus and also contributes to the extraembryonic membranes. It is the blastocyst stage when these two cell lineages are differentiated for the first time. The formation of the developmentally competent bovine blastocyst involves the process of cell division, differentiation and cell death. It is known that cell death, in the early embryos, occurs by apoptosis, which presumably serves to eliminate unwanted cells during the critical changes that take place in early embryonic development (Watson et al., 2004; Watson et al., 1999; Zernicka-Goetz, 2005).

Before implanting in the uterine epithelium, the blastocyst must hatch from its enclosing zona pellucida with species-specific mechanisms. When an expanded blastocyst gets bigger, eventually the stretched zona pellucida develops a crack. The expanded blastocyst escapes by a process called hatching. In some species (e.g. hamster), embryos produce their own enzyme to digest a hole in the zona pellucida, and then the blastocyst hatches (Mishra and Seshagiri, 2000). Not all the expanded blastocysts are able

to hatch, so ability of hatching is known to be one of the indicators for embryo quality (Gordon, 2003). The trophectoderm initiates implantation through direct contact to the uterine endometrium and contributes the extraembryonic membranes. In the bovine, this hatched blastocyst elongates rapidly to fill the uterine lumen (Bavister, 1995).

Apoptosis in Preimplantation Embryos

Cell death is observed as a normal feature of both *in vitro* and *in vivo* mammalian embryos. There are two forms of cell death, necrosis and apoptosis. Necrosis usually occurs in a group of neighboring cells, and it is characterized by cellular swelling and membrane rupture. In contrast, apoptosis, also called programmed cell death, in embryos affects single cells without damage to surrounding cells. It involves several well-characterized morphological features including chromatin condensation, DNA fragmentation, and membrane blebbing (Fabian et al., 2005; Hardy, 1997). Fragmented DNA can be visualized by the appearance of a DNA ladder pattern on agarose gel electrophoresis. However, due to the presence of the small number of cells in mammalian preimplantation embryos, the gel electrophoresis DNA fragment detection method is impractical (Jurisicova et al., 1998; Jurisicova et al., 1996; Neuber et al., 2002). The most frequently used method of apoptosis detection is the terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay. It allows *in situ* assessment of DNA fragmentation in the nucleus of the embryo. TdT catalyzes the incorporation of fluorescein-12-dUTP at 3'-OH DNA ends of fragmentation. This

fluorescein-12-dUTP labeled DNA can be detected by fluorescent microscopy (Fedorcsak and Storeng, 2003; Neuber et al., 2002).

The presence of apoptosis has been observed in blastocysts from mouse, human, and bovine obtained *in vivo*, as well as in embryos produced *in vitro*. It is suggested that apoptosis is likely to be a required feature of normal preimplantation development. A possible function of apoptosis is the elimination of abnormal cells, damaged cells, or a sub-lineage of cells with an inappropriate developmental potential as a quality control mechanism (Fabian et al., 2005). Thus, a level of apoptosis in embryonic cells can be used as an indicator of embryo quality and viability. Several studies have shown that in the bovine, apoptosis starts to show from the 8 -cell stage *in vitro* and the 21-cell stage *in vivo*, and a higher level of apoptotic cells is observed in blastocysts derived *in vitro* than *in vivo* (Gjorret et al., 2003; Hardy, 1997; Pomar et al., 2005). Therefore, the level of apoptosis during *in vitro* production of embryos could be related to inefficient oocyte maturation, fertilization, and culture conditions. The exposure to damaging factors, such as oxygen free radical, UV irradiation, or heat shock could increase the incidence of apoptosis in early embryos (Jurisicova et al., 1996; Long et al., 1998). It has been documented previously that heat stress can induce TUNEL- and Caspase-positive apoptotic cells at the late 8- to 16-cell stages in the bovine embryos (Paula-Lopes and Hansen, 2002).

In addition, Brison and Schultz (1997) and Yuan et al. (2002) reported that the degree of apoptosis in blastocysts is dependent on the embryo density of *in vitro* culture. The embryos cultured individually have a higher percentage of apoptotic cells labeled

with TUNEL than those embryos cultured in groups of 25 in 50 µl drops, and blastocyst formation and hatching rates of single-cultured embryos were significantly reduced. Studies have shown that these high incidents of apoptosis and reduced developmental rates can be reversed by supplementation of the culture medium with certain growth factors such as epidermal growth factor (EGF), transforming growth factor α or β1 (TGF-α or TGF- β1) (Hardy, 1997; Paria and Dey, 1990). From these results, damaged cells of embryos can be reduced by growth factors, which eventually help embryonic development.

Bovine *In Vitro* Embryo Production

Bovine *in vitro* embryo production (IVP) has been developed and widely used as a research tool for basic science research in fertilization and embryogenesis as well as applications in animal biotechnology such as embryo transfer, embryo sexing, and cloning (Galli et al., 2003; Lohuis, 1995). These animal biotechnologies have potential roles in species conservation, selective breeding, and infertility treatment (Hansen, 2006; Thompson et al., 2007). Therefore, the successful development and application of these biotechnologies are critically dependent on the establishment of IVP. IVP of bovine embryos is composed of three biological steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). In routine bovine IVP, about 90% of oocytes undergo nuclear maturation and about 70 to 80% undergo fertilization and cleavage to at least the 2-cell stage. The rates of development to the blastocyst stage are limited to about 30 to 40% following IVM and IVF (Lonergan et al., 2001). Many

different protocols, media, and chemicals used for IVP of bovine embryos have been developed and studied among different laboratories. This overview describes procedures and culture systems generally used for the IVP of bovine embryos that are used by many laboratories. Additionally various components of the embryo *in vitro* culture method, such as the volume of culture medium, oil overlay, or serum supplementation are reviewed in this section.

In Vitro Maturation

The maturational competence of oocytes is influenced by the size of follicles from which the cumulus-oocyte complexes (COC) are harvested. When using oocytes collected from ovaries of slaughtered cattle, COCs that are recovered from > 6 mm diameter follicles have been proven superior in developmental competence to those from smaller follicles (Lonergan et al., 1994). Bovine oocytes are very sensitive to temperature fluctuations, so it is important to maintain the temperature as close to 39°C as possible during the collection procedure. The complex culture medium TCM-199 supplemented with 10% fetal calf serum (FCS) or 6 mg/ml bovine serum albumin (BSA) as a protein source and gonadotropins (FSH, LH) has been the most widely used medium for bovine oocyte maturation (Bavister et al., 1992; Leibfried-Rutledge et al., 1986; Sirard et al., 1998). The volume of the medium used for IVM can be 500 µl to 1ml in the test tubes without oil overlay and 4-well dishes or 10 µl to 100 µl microdrops under mineral oil in the petri dishes. The maturation of bovine oocytes is completed with extrusion of the first polar body after 20-24 hours of incubation in 5% CO_2 in air at 39°C. If the slaughtered

donors are located far away from the main laboratory, maturation can be completed during transport from the location of oocyte collection to the laboratory in tightly closed glass tubes in portable incubators with only temperature requiring regulation.

In Vitro Fertilization

Usually frozen sperm are used for bovine *in vitro* fertilization (IVF), swim-up and Percoll gradient centrifugation are commonly used methods for separating motile sperm after thawing. Parrish et al. (1995) showed that the Percoll gradient centrifugation was superior to the swim up method in recovery of the motile sperm. However, recent studies have reported that some batches of Percoll have an endotoxic effect, thus it has been discarded for use in assisted reproduction techniques in human (Gordon, 2003; Marquant-Leguienne and Humblot, 1998). There are now several commercially available alternatives to Percoll. For instance, BoviPure™ is a sperm separation and purification product formulated specifically for bull sperm (Samardzija et al., 2006). Two media are generally used for bovine IVF: a TALP (Tyrode albumin lactate pyruvate)-based medium or a SOF (Synthetic oviductal fluid)-based medium supplemented with 6 mg/ml of BSA as a protein source. Various concentrations of penicillamine, hypotaurine, and epinephrine (PHE), which have beneficial effects on bovine sperm motility and acrosome reaction, are added to IVF medium (Miller et al., 1994a). Furthermore, the addition of 10 μg/ml of heparin in the TALP medium is known to increase sperm penetration rates (Parrish et al., 1986). The concentration of spermatozoa to obtain the maximum level of fertilization with minimal polyspermy needs to be assessed by performing IVF tests with

different sperm concentrations. The fertility of the bull can influence fertilization and embryo development rates. Performing IVF with the semen from a single bull of known fertility is an efficient way to get low variation in blastocyst yields from batch to batch (Marquant-Leguienne and Humblot, 1998). Bovine IVF is completed overnight after 18-20 hours of co-incubation of sperm and oocytes. At this time, the remaining cumulus cells on bovine oocytes and spermatozoa are completely removed. These presumptive zygotes are transferred to a culture system appropriate for bovine embryo development.

In Vitro Culture

In vitro culture (IVC) is the development of fertilized oocytes to the blastocyst stages following maturation and fertilization. There are many different types of protocols and methods for bovine *in vitro* culture including various co-cultures with various cell types and cell-free systems (Galli et al., 2003). Microdrop, tube, and four-well methods have been commonly used in many sutides on bovine IVC. Different culture dishes with various volumes of culture medium and numbers of embryos, and in the presence or absence of oil overlay in IVC are major factors influencing development of bovine embryos (Brinster, 1968; Gardner and Lane, 2000). Recently, Well of the Well (WOW) (Vajta et al., 2000), Glass Oviduct (GO) (Thouas et al., 2003), and Microchannel (Raty et al., 2004) culture methods have been developed, and these new culture methods have proven to be better culture environment and more closely mimic the *in vivo* environment than the traditional culture methods. Moreover, several studies have been shown that the surrogate sheep oviduct can be used to produce high quality blastocysts from the *in vitro*

matured and fertilized embryos (Enright et al., 2000; Lonergan et al., 2003; Rizos et al., 2002). SOF is commonly used media for bovine *in vitro* embryo culture. The medium is originally based on the biochemical analysis of ovine oviductal fluid (Gordon, 2003). This medium has been modified by addition of amino acid (minimal essential medium (MEM) and basal medium eagle (BME)) (Takahashi and First, 1992) and usually supplemented with fatty acid free BSA. Most of these media contain serum as a protein source. Several laboratories have demonstrated a favorable effect of serum on the development of bovine embryos in defined or BSA containing media. Embryo cleavage rate is usually assessed on day 2 pi. The developing embryos should have reached at least the early blastocyst stage at day 7 pi, the majority being at the blastocyst stage, and some at the expanded and hatched blastocyst stages at Day 8 pi and Day 9 pi respectively.

Serum

Serum is frequently added to culture medium as a protein source in mammalian embryo *in vitro* culture. Several laboratories have demonstrated that the presence of serum in bovine embryo culture media increases the percentage of fertilized oocytes reaching the blastocyst stage. Serum appears to enhance the total cell number and hatching rate in blastocysts (Lim et al., 1994; Van Langendonckt et al., 1997; Yoshioka et al., 1997). Serum may provide beneficial factors to embryo culture such as sufficient energy substrates, amino acids, vitamins, growth factors, and antioxidants (Okada et al., 2006; Pinyopummintr and Bavister, 1991; Van Langendonckt et al., 1997). Because of these advantageous effects, serum is widely used as a supplement in various culture

media. However, serum is a pathological fluid formed by blood clotting, which may induce chemical alterations with possible harmful effects for embryo culture. In addition, the composition of serum is highly variable from different suppliers and even between batches or lots (Bavister, 1995; Maurer, 1992). Van Langendonckt et al. (1997) observed that some batches of serum decreased the blastocyst rates and cell numbers and increase apoptotic cells. Furthermore, serum supplementation is known to alter both embryo morphology and kinetics within *in vitro* culture (Holm et al., 2002). Several cinematographic analysis studies have shown that serum accelerates embryo developmental rate *in vitro* between the 9-16 cell and morula stages. This kinetic change causes premature blastulation, which is characterized by poor compaction of morulae when comparing *in vivo* and *in vitro* morulae cultured in medium supplemented with serum (Holm et al., 2002; Van Langendonckt et al., 1996; Yoshioka et al., 1997). Additionally, studies have shown that embryos produced in serum-containing media contain more lipid than those produced in media without serum. The embryos cultured with serum-containing medium are also less likely to survive cryopreservation (Abe and Hoshi, 2003; Bavister, 1995). Therefore, serum improves embryo development rates and kinetics but affects embryo quality. Another feature of serum is that it inhibits the early cleavage divisions but promotes morula and blastocyst development. Therefore, it is routinely added around the 8-16 cell stage of *in vitro* culture (Bavister, 1995; Van Langendonckt et al., 1997).

Embryo Density

Embryo density (ratio of embryos to volume of culture medium) has been proven to be an important factor for *in vitro* culture of preimplantation embryos. Studies show high rates of embryo development by increasing the embryo density. There have been reports showing that embryos cultured in a small volume of medium and in groups have a higher blastocyst rate, hatching rate, and cell number and a lower incidence of apoptosis than those cultured individually in the mouse (Brison and Schultz, 1997; Canseco et al., 1992; Kato and Tsunoda, 1994; Salahuddin et al., 1995), sheep (Gardner et al., 1994), and bovine (De Oliveira et al., 2005; Keefer et al., 1994; Palasz and Thundathil, 1998; Yuan et al., 2002). Paria and Dey (1990) demonstrated that embryos secrete specific growth factors that may act in an autocrine or paracrine manner. There has been evidence that such an inferior embryo development rate and quality of individually cultured embryos could be improved by addition of EGF or TGF- α and TGF- β1 (Hardy, 1997; Khurana and Niemann, 2000; Paria and Dey, 1990). In addition, Fujita et al. (2006) reported that culturing bovine embryos in conditioned medium derived from a group of other embryos increased the blastocyst formation rates of single cultured embryos. Thus, the beneficial effects of a high embryo density during *in vitro* culture may have been originated from the embryos themselves. Another assumption is that increasing embryo density may deplete embryo toxic substances in the culture medium or reduce the concentrations of naturally produced inhibitors like glucose (Bavister, 1995).

Oil

Silicone, paraffin, and mineral oil are routinely used to overlay in microdrop culture methods of *in vitro* culture. Oil overlay prevents a small volume of culture medium from rapid evaporation which causes changes in component concentration or osmolarity in the culture medium. Additionally, it helps to reduce temperature and pH fluctuations during examination or manipulation of the embryos (Boatman, 1987; Brinster, 1968; Tae et al., 2006). Moreover, Tae et al (2006) showed that use of high quality sterile-filtered light paraffin oil increased embryo development rates by absorbing toxic materials from the embryo culture medium when compared to use of the light mineral oil in the culture medium. However, oil can be a source of toxic contamination in the embryo culture. Provo and Herr (1998) reported that washed light mineral oil exposed to direct sunlight for 4 hours inhibited mouse embryo development. The toxic elements have been reported in certain types and batches of oil and those cultured with contaminated oil overlay have proven to be harmful for embryonic development (Erbach et al., 1995; Otsuki et al., 2007; Van Soom et al., 2001). Therefore, the selection of oil and prescreening batches and lots of oil are important for successful *in vitro* production.

Microgravity and Effects on Embryo Development

Microgravity

Gravitational force is the attraction between any two masses with a force inversely proportional to the square of the distance between them (Morey-Holton, 2003).

When one mass is significantly larger than the other, like Earth, the gravitational force is most apparent. About 90% of gravitational force at the surface of Earth is still present in a spacecraft orbiting between the altitude 120 miles and 360 miles above Earth (Klaus, 2001; Committee on Space Biology and Medicine, 1998). However, if the spacecraft is accelerated in orbit to be equal in the amount of gravitational force and centrifugal forces, but opposite to their vector, near weightlessness can be achieved (Hemmersbach et al., 2006; Committee on Space Biology and Medicine, 1998). Depending on motion and acceleration, the orbital spacecraft generates a gravity range of 10^{-5} to 10^{-2} (Morey-Holton, 2003), which is referred to as microgravity. Microgravity means simply a condition in which some of the effects of gravity are reduced compared to the 1g on the Earth (Klaus, 2001).

Life on the Earth has been influenced by a gravitational force throughout time as we know it. The physiology, shape, function, and behavior of all the species on the Earth have been affected by this constant force (Briegleb, 1992; Morey-Holton, 2003). Microgravity conditions may have a detrimental effect on physical and biological processes in organisms on the Earth. Various changes in physical factors occur in microgravity conditions. The weight is decreased, and the effect that gravitational force has on the convection, sedimentation, and hydrostatic pressure on the Earth is also reduced in microgravity (Committee on Space Biology and Medicine, 1998; White and Averner, 2001). Studies conducted during and after spaceflight have reported significant physiological changes in astronauts and experimental animals (Hughes-Fulford, 1991; Planel, 2004; Tischler and Morey-Holton, 1993; West et al., 1982; Whedon, 1982; White,

1998; White and Averner, 2001). These changes include loss of fluids and electrolytes, cephalad fluid shifts, loss of muscle mass, decreased pulmonary function, a modified immune response, and loss of calcium and mineralized bone. In addition, Hughes-Fulford (1991) in a review demonstrated that cells could respond to changes in gravity, and that gravitational stress can alter metabolic activities and disturb the basic cellular and metabolic functions even in a single cell. Experiments on various cell types exposed to microgravity conditions have shown that the microgravity conditions caused decreasing cell proliferation, loss of mitochondrial activity, changes in subcellular organization and cytoskeleton, and induction of apoptosis (Bucaro et al., 2004; Cogoli et al., 1984; Hughes-Fulford, 1991; Lewis, 2004; Lewis et al., 1998; Yang et al., 2002). For instance, microgravity can slow down the development of bone cells. Specifically, the total number of osteoblasts has been found to be significantly reduced in space experiments. And, culturing bone cells in microgravity conditions causes reduced glucose utilization and prostaglandin synthesis and a significantly altered cytoskeleton (Hughes-Fulford and Lewis, 1996). In addition, Lewis et al. (1998) reported abnormalities in cytoskeletal morphology and increased apoptotic cells in space flown Human T lymphoblastoid cells (Lewis et al., 1998).

Researchers found that microgravity conditions are actually advantageous for mammalian tissue growth (Duray et al., 1997; Freed et al., 1999; Unsworth and Lelkes, 1998; Vunjak-Novakovic et al., 2002). Typically cells are grown *in vitro* in a flat and two-dimensional layer on the bottom of a petri dish. In a living organism, however, cells grow and form three-dimensional layers of tissue consisting of specialized and

differentiated cells. Cells grown *in vitro* in petri dishs do not differentiate, and they are essentially useless in biomedical applications such as tissue transplants. Studies have proven that mammalian cells grown in microgravity conditions form tissues that more closely resembled *in vivo* cells (Freed et al., 1997; Jessup et al., 2000; Unsworth and Lelkes, 1998). Due to the lack of convective fluid mixing and sedimentation processes that require gravity, the cells do not settle to the bottom of the culture container; rather, they are suspended in the media. Cell suspensions aggregate and yield higher cell densities than on the ground and form three-dimensional tissue aggregates that mimic human tissues. This tissue culture provides excellent models in the pharmaceutical and organ transplantation fields (Duray et al., 1997; Unsworth and Lelkes, 1998).

Simulated Microgravity on Earth

Space flight studies offer a unique opportunity to determine the effects of microgravity on biological systems. Experimentation during space flight should be well controlled in order to improve our understanding of microgravity. However, there are many challenges and limitations when conducting biological experiments during a space flight. First, the experimental equipment for on board investigations needs to be compact, light, and simple because of the limited space available in the fight. Therefore, only relatively simple experimental procedures can be performed (Lynch and Martin, 2005; Sundaresan et al., 2004). Moreover, space flight opportunities are very limited and of short duration due to high costs. In addition, space flight is associated with other effects of various non-gravitational factors, such as radiation, stress, atmospheric pressure,

launch and landing of the flight and other aspects. Thus, these difficulties of the space flight experiment have led to the development of ground-based models that simulate microgravity (Halstead, 1982; Klaus, 2001; Morey-Holton and Globus, 2002; Tou et al., 2002; Vunjak-Novakovic et al., 2002).

On the Earth, real microgravity conditions can be produced by a free fall from drop towers, parabolic flights of airplanes, and sounding rockets. However, because these methods create very short periods of the simulated microgravity, it is difficult to determine the apparent alterations of the physiology and function in humans, animals, or plants (Hemmersbach et al., 2006; Hoson et al., 1997; Lynch and Martin, 2005). The ground-based models for the simulating microgravity exposure in humans are head-out water immersion (HOWI) and bed-rest with a 6° head-down tilt (Lynch and Martin, 2005; Planel, 2004). These techniques induce shifts in body fluids towards the head, shifts of the abdominal organs towards the chest, and minimize the hydrostatic gradient within the cardiovascular system similar to those experienced during a space flight (Morey-Holton and Globus, 2002; Planel, 2004). In animal studies, the hindlimb suspension model (HLS) is frequently used to examine the effects of the microgravity on bones (Doty and Morey-Holton, 1982) and muscles (Templeton et al., 1982). Rats are suspended by their tail with the head-down position at a 30 degree angle. Only their hind limbs are unloaded while the forelimbs are remaining free to turn 360° and search the entire surface of its cage for food. These experiments have also shown that unloading of the hind limbs on the Earth is similar the effects seen during the first week of spaceflight (Morey-Holton and Globus, 2002; Planel, 2004; Tou et al., 2002).

Clinostats are used to study the changes occurring at the cellular level in plants and animals in the simulated microgravity. One major feature in the microgravity environment of space is the lack of sedimentation. On the ground, this situation can be created by rotating particles about the horizontal axis in suspension. The clinostats rotate the cells on a horizontal axis and develop the solid phase rotation to maintain cells in suspension, where they experience randomized gravitational vectors (Hemmersbach et al., 2006; Klaus et al., 1998). The combined actions of the centrifugal force, gravity and the viscosity of the medium result in maintaining cells in suspension (Gerecht-Nir et al., 2004; Hammond and Hammond, 2001; Klaus, 2001). A schematic representation of the principle of clinostat rotation is shown in Figure 2.1. There are different clinostats in their rotational speed: slow-rotating clinostats are rotated with 1-2 rpm (rotation per minute), fast-rotating clinostats with 50-100 rpm (Cogoli, 1992). The clinostat that rotates around one axis perpendicular to the gravitational force is a 2-dimensional clinostat. A 3-dimensional clinostat rotates around two rotation axes oriented at right angles to each other (Hemmersbach et al., 2006; Planel, 2004)

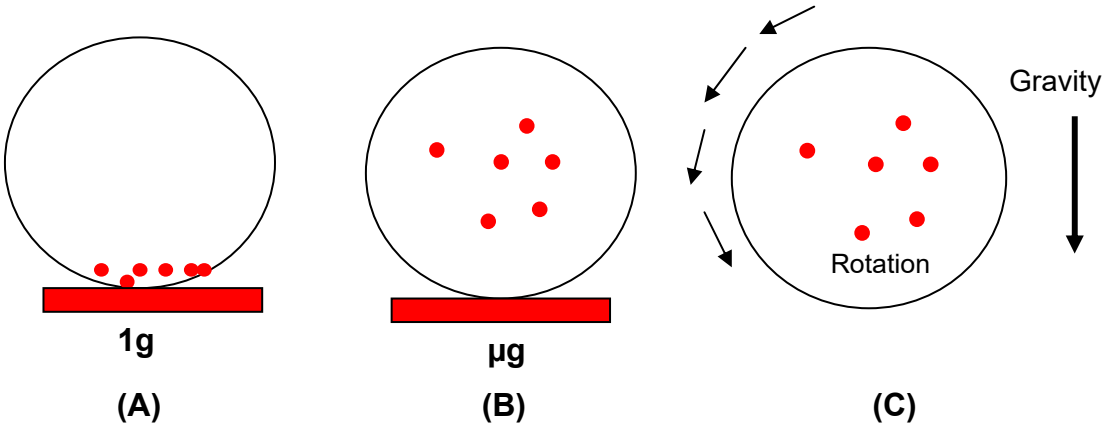

Figure 2.1 Schematic Representation of the Principle of Clinostat Rotation.

Note: (A) particles sediment if their density is higher than medium in the container on the ground. (B) particles are in suspension due to the lack of sedimentation in microgravity condition. (C) .the rotation of clinostat maintains the particles in suspension.

The NASA biotechnology group at Johnson Space Center has developed the Rotary Cell Culture System (RCCS) ™ that is functionally a similar device to that of clinostats (Duray et al., 1997). It has become a useful tool to determine the effects of microgravity on a variety of mammalian *in vitro* cell cultures and tissue culture on the ground (Freed and Vunjak-Novakovic, 1997; Goodwin et al., 1993; Guidi et al., 2002). Studies have shown that changes observed in cell morphology and functions in the RCCS bioreactor experiments have proven to be very similar to those observed after space flights (Goodwin et al., 1993; Ingram et al., 1997; Schwarz et al., 1992; Unsworth and Lelkes, 1998). RCCS™ consists of a power supply, a High Aspect to Ratio Vessel (HARV), and a rotator base on which the vessel is rotated (shown in Figure 2.2.). The HARV, which is completely filled with medium, is rotated about the horizontal axis at a

constant velocity. The combined actions of the centrifugal force, gravity and the viscosity of the medium create the condition of maintaining cells in suspension (Hammond and Hammond, 2001; Klaus, 2001). The gas exchange is achieved by diffusion through a semi-permeable membrane at the back of the HARV (Lynch and Martin, 2005; Unsworth and Lelkes, 1998). Additionally, it has been shown that the RCCS bioreactor with HARV generates a low shear simulated microgravity of about 10^{-2} g (Unsworth and Lelkes, 1998).

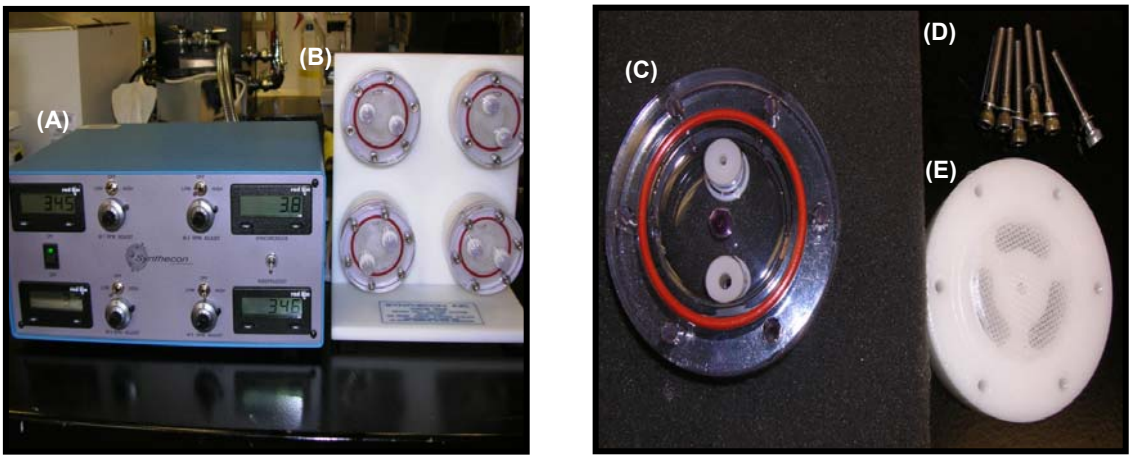

Figure 2.2 The Rotary Cell Culture System™ and High Aspect Ratio Vessel™ (HARV); Manufactured by Synthecon, Inc. (Houston, Texas).

Note: (A) power supply, (B) rotator base, (C) front plate of HARV, (D) HARV peripheral screws, and (E) back plate of HARV with semipermeable membrane.

Effects of Microgravity on Embryo Development

Since the development of living organisms on the Earth is constantly influenced by gravity, it is hypothesized that microgravity conditions may alter fertilization and

embryogenesis in an organism's development (Ronca, 2003). For several decades, experiments have been performed to determine the possibilities of normal embryo development during the space flight and in simulated microgravity conditions. A variety of animal models have been used, such as medaka fish (Ijiri, 1998), zebra fish (Gillette-Ferguson et al., 2003; Shimada and Moorman, 2006), sea urchin (Marthy et al., 1994), salamander (Gualandris-Parisot et al., 2002), mouse (Kojima et al., 2000; Schenker and Forkheim, 1998), and rat (Serova and Denisova, 1982), in studies carried out in space flight and simulated microgravity on the Earth to determine the effects of microgravity on embryo development.

All of the experiments with fish and amphibians flown during the process of fertilization and embryo development showed a similar pattern that their fertilization and development could occur in space and in simulated microgravity conditions even though there were some degrees of cellular and molecular alterations. In 1994, medaka fish successfully mated and laid eggs during International Microgravity Laboratory (IML)-2 mission in 1994 (Ijiri, 1998). Fertilization and hatching rates were normal compared to ground based control, and this study also showed primordial germ cell migration in live offspring occurred normally in space. In addition, studies have shown that the zebra fish embryos were able to develop in simulated microgravity experiments; however, researchers have found the abnormalities in developing organ systems and alterations in gene expression for heat shock proteins in the zebra fish compared to controls (Gillette-Ferguson et al., 2003; Shimada and Moorman, 2006; Shimada et al., 2005). Sea Urchin eggs were fertilized under microgravity conditions during sounding rocket flights

(Marthy et al., 1994). Their fertilization occurred normally without any alterations in the sperm-egg recognition and attachment processes, the polarization of the egg, and the establishment of an animal-vegetal morphogenetic gradient. In the salamander, even though some cellular alterations occurred at the time of the fertilization and early cleavage stage during space flight, embryos developed to the adult stage (Aimar et al., 2000; Gualandris-Parisot et al., 2002). In a similar pattern, female frogs were sent to the space and induced to ovulate eggs that were artificially inseminated (Souza et al., 1995). Frog embryos produced in the microgravity showed slight displacement of the mitotic asters toward the vegetal pole at the 2-cell stage and also abnormalities in blastula and gastrula morphogenesis. Nevertheless, the embryos were able to regulate and develop into normal tadpoles and they were matured into normal frogs.

In mammalian studies, several experiments have been conducted mostly in the rat and mouse early embryos in space and in simulated microgravity conditions, and results have not been consistent. The effect of simulated microgravity on oocyte maturation in the mouse was studied by Wolgemuth and Grills (1984). At several rotational speeds ranging from ¼ to 100 rpm, the meiotic progression to metaphase II occurred normally except at 100 rpm, where a significant decreased in oocyte maturation rates was observed. The effect of simulated microgravity on *in vitro* fertilization was investigated in the mouse (Kojima et al., 2000). When mouse oocytes were rotated at 100 rpm in the presence of sperm, the oocytes were fertilized normally. However, in this study, there was a statistically significant decrease in the number of embryos reaching the morula and blastocyst stages after 96 hours in culture under the clinostat rotation at 100 rpm (Kojima

et al., 2000). In addition, Rappolee et al. (2004) showed mouse embryo lethality after 24 hours of culture in a Rotary Cell Culture System™ (RCCS). Moreover, 2 cell mouse embryos were cultured during a flight of the space shuttle Columbia (STS-80). In this experiment, none of the two-cell embryos developed to further stages (Schenker and Forkheim, 1998). In 1982, rats were mated in a Cosmos 1129 biosatellite; and although 2 out of 5 females experienced fertilization and early embryo development, the gestation was discontinued (Serova and Denisova, 1982).

Several studies have investigated the influence of gravity changes on the function of mammalian sperm. Human sperm motility similarly has been examined in the microgravity environment using the clinostat and parabolic flight models. There was no significant difference in sperm motility between the control and clinostat rotation, however sperm motility in the parabolic fight experiment was decreased (Ikeuchi et al., 2005). Additionally, Andrews and Winters-Hilt (2004) reported that bovine sperm lost their motility within 3 hours at 9 rpm RCCS™ rotation. Also, they observed abnormal sperm nuclear membranes and mitochondria in sperm under simulated microgravity conditions compared to those in a control group (Andrews and Winters-Hilt, 2004). In contrast, Engelmann et al. (1992) showed that bovine sperm motility was increased in sounding rockets used to simulate microgravity.

CHAPTER III

THE EFFECT OF CULTURE METHODS AND SERUM SUPPLEMENTATION ON DEVELOPMENTAL COMPETENCE OF BOVINE EMBRYOS CULTURED *IN VITRO*

Abstract

The objective of this study was to compare the developmental competence of bovine *in vitro* fertilized embryos in three different culture methods; microdrop method (50 µl of medium under mineral oil in petri dishes) compared to tube methods (1 ml of medium in tubes) with or without oil overlay, and to examine the influence of fetal bovine serum (FBS) in culture methods. A total of 1,696 presumptive zygotes were randomly allocated to one of following culture conditions: (Group 1) microdrop method supplemented with 10% (v:v) FBS at day 5 post-insemination (pi); (Group 2) microdrop method without FBS; (Group 3) tube method with oil overlay and FBS added at day 5 pi; (Group 4) tube method with oil overlay; (Group 5) tube method without oil overlay but FBS added at day 5 pi; (Group 6) tube method without oil overlay and FBS. There were no differences ($P > 0.05$) in cleavage rates among the culture methods (78.3%, 75.6%, and 74.9% for microdrop, tube with oil overlay, and tube method without overlay, respectively). However, regardless of serum addition, blastocyst rates in the microdrop method (30.5%) were significantly higher ($P < 0.05$) than those in the tube method

without oil overlay (9.8%). There was no difference ($P > 0.05$) between with or without serum in blastocyst rate regardless culture methods. Numerically, the highest blastocyst rate was observed in the microdrop method with the FBS supplementation (Group 1; 36.6%). Overall, the microdrop method was the optimum culture method among the culture methods; however, serum supplementation did not significantly affect the blatocyst rate.

Introduction

Bovine embryo *in vitro* production (IVP) has become an important tool for basic science research and for applications in animal biotechnologies (Galli et al., 2003; Lohuis, 1995). In support of this, a diverse array of *in vitro* culture methods (e.g., microdrop, tube, and four well methods) have been developed and studied among different laboratories (Rombauts and Wood, 2000). In these component based IVP systems, bovine preimplantation embryos have been cultured from the zygote to blastocyst stages in various volumes of culture medium, and in the presence or absence of oil overlay with different culture methods, and serum has been frequently added to culture medium as a protein source for *in vitro* culture (IVC) (Bavister, 1995). Since Brinster first developed the microdrop culture method (Brinster, 1963), this culture method has become the most commonly used culture method among IVF laboratories (Boatman, 1987; Nagy et al., 2003). The microdrop method is achieved by culturing a group of embryos in a small volume (10 to 100 µl) of culture medium covered with liquid oil (e.g. mineral, paraffin, or silicon oil) that prevents the microdrop of medium from rapid evaporation and controls

the pH changes of the culture medium (Brinster, 1968; Miller et al., 1994b; Tae et al., 2006). Nevertheless, some studies have shown that oil overlay of culture medium results in interactions with steroid hormones such as oestradiol and progesterone present in the medium causing alterations in medium composition (Miller and Pursel, 1987; Shimada et al., 2002). Additionally, some batches or lots of oil in this method can be a source of toxic contaminants in embryo culture (Erbach et al., 1995; Otsuki et al., 2007; Provo and Herr, 1998; Van Soom et al., 2001). In order to avoid oil use, a group of embryos could be cultured in a relatively large volume (1-3 ml) of culture medium in either culture tubes or 4-well dishes. Culturing embryos in a large volume of medium, however, potentially has an adverse effect. It may dilute embryo-derived growth factors which may have beneficial effects on their development (Canseco et al., 1992; O'Neill, 1998).

Additionally, serum supplementation of the culture medium during the later stage (morulae or blastocyst stage) of embryo development is known to increase blastocyst and hatching rates and cell numbers of bovine embryos (Lim et al., 1994; Pinyopummintr and Bavister, 1991; Van Langendonckt et al., 1997; Yoshioka et al., 1997). However, the composition of serum is highly variable from different suppliers and even between batches or lots (Maurer, 1992). Moreover, some batches of serum contain non-defined molecules that could be detrimental to embryo development (Bavister, 1995).

These components of *in vitro* culture systems used for IVP influence the developmental competence of mammalian embryos (Bavister, 1995; Galli et al., 2003; Gardner and Lane, 2004). For this reason, success rates of the embryo culture vary among different protocols, and the efficiency of culture methods also differs between

laboratories and studies. Furthermore, a recent review by Gardner (2008) emphasized the necessity of prescreening batches and lots of serum in order to optimize the efficacy of culture systems (Gardner, 2008). It is therefore important to examine these components and culture methods affecting the development of *in vitro* derived embryos when applying routine IVP to new studies. In this study, we designed a simple and comparative experiment evaluating different culture methods and effects of serum on bovine embryo development. The developmental competence of bovine embryos were compared between the microdrop method with 50 µl of culture medium covered with mineral oil and the tube methods with 1 ml culture medium (with or without mineral oil overlay), which contained an equivalent number of embryos, and to examine the influence of serum supplementation on embryos in *in vitro* culture.

Materials and Methods

All the chemicals utilized in the present investigation were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise indicated. Bovine cumulus-oocyte complexes (COCs) were purchased from a commercial supplier (BOMED, INC., Madison, WI). Each week, approximately 600 bovine oocytes were shipped in 2 ml of the maturation medium in tightly closed tubes and were over-night shipped in a 39°C portable incubator. On arrival at the laboratory, COCs were removed from the shipping unit and were incubated at 39°C and 5% CO_2 in air for 24 hours.

Sperm Preparation and *In Vitro* Fertilization

The sperm preparation for IVF used the BoviPure™ gradient and was performed according to the protocol provided by the manufacturer as follows (Nidacon International AB, Gothenburg, Sweden). All the media used for sperm preparation were warmed at 39°C before use. In a 15 ml conical centrifuge tube, 2 ml of BoviPure™ Bottom Layer was placed and then carefully layered with 2 ml of BoviPure™ Top Layer on the top of the BoviPure™ Bottom Layer to make a gradient. Two straws of frozen semen from a single Angus bull were thawed in a 37°C water bath for 120 seconds. The thawed semen were gently mixed with BoviPure™ Extender and brought to a 2 ml volume. The 2 ml of the prepared semen was then gently layered on top of the BoviPure™ gradient and centrifuged for 20 min at 500 × *g*. After the centrifugation, the fluid above the sperm pellet was carefully removed with a sterile Pasteur pipette. The pellet was resuspended with 5 ml of BoviPure™ Wash and centrifuged for 5 minutes at 300 × *g*. This pellet was resuspended again in 5 ml of warmed IVF-TL (Tyrode's Albumine Lactate Pyruvate) medium (Millipore, Billerica, MA), and centrifuged for 5 minutes at 300 × *g*. IVF-TL medium was supplemented with pyruvate (0.2 mM), fatty-acid-free Bovine Serum Albumin (BSA-FAF, 6 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), heparine 10 µg/ml, hypotaurine 0.5 µg/ml, and epinephrine 0.5 µg/ml (Miller et al., 1994a). The number of sperm was counted using a hemocytometer, and the sperm pellet was diluted to a concentration of 50 x 10^6 spermatozoa/ml. To make the 2 x 10^6 final concentration, 2 µl of diluted sperm were added to each 50 µl drop of the fertilization medium (IVF-TL) containing 10 matured oocytes that had been washed previously in the

fertilization medium (IVF-TL). Sperm and oocytes were co-incubated at 39°C and with 5% CO_2 in air for 18 hours.

In Vitro Culture

Following fertilization, presumptive zygotes were denuded from cumulus cells by vortexing for 2 minutes and washed 3 times with HEPES-TL wash medium (Bioniche, Pullman, WA). They were cultured in Synthetic Oviductal Fluid (SOF) culture medium (Millipore, Billerica, MA) supplemented with pyruvate (0.4 mM), 100x MEM (20 µl/ml), 50x BME (10 µl/ml), BSA-FAF (8 mg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 39°C and with 5% CO_2 in air (Edwards et al., 1997). All the culture medium was equilibrated at 39°C and with 5% CO_2 in air overnight either in the 35 x 10 mm sterile polystyrene disposable Petri dishes (Fisher Scientific, Pittsburgh, PA) or 12 x 75 mm sterile polystyrene disposable tissue culture tubes (Fisher Scientific, Pittsburgh, PA) before use. The sterile mineral oil (Cat. No. M5310; Sigma) was used for the culture, and it was stored in the dark to avoid the production of embryo-toxic compounds (Provo and Herr, 1998). The 10% (v:v) of FBS (Fetal Bovine Serum, Gibco/Invitrogen, Carlsbad, CA) was added to selected treatment groups in the culture medium at day 5 pi (post insemination) in the culture (Lim et al., 1994). All the FBS and mineral oil that was used in the present study were from one batch (lot).

Experimental Designs

To evaluate the effects of culture methods and serum supplementation in bovine *in vitro* culture, three different culture methods (microdrop method, tube method with oil overlay, and tube method without oil overlay) and with and without serum supplementation were used in a 3 X 2 factorial experiment. A total of 1,696 presumptive zygotes were randomly distributed to the following six groups. A group of approximately 50-70 presumptive zygotes were cultured in Group 1: microdrop method (50 µl microdrops of culture medium covered with mineral oil in the petri dish) and with serum supplementation (10% (v:v) FBS added at day 5 pi); Group 2: microdrop method without serum supplementation; Group 3: tube method (1 ml of culture medium in the test tube) with mineral oil and serum supplementation; Group 4: tube method with oil overlay but no FBS supplementation; Group 5: test tube method without oil overlay and FBS added at day 5 pi; Group 6: tube method without oil overlay and no serum supplementation. The cleavage rate of the embryos was assessed and the culture medium in all groups was replaced with the fresh culture medium at day 2 pi. The blastocyst development was assessed at day 9 pi.

Statistical Analysis

Experiments were repeated three times and data were analyzed by two-way ANOVA (culture methods and serum supplementation and their interation). All percentage data were subjected to arcsine transformation before statistical analysis. Data are expressed as mean ± SEM. A probability of $P < 0.05$ was considered to be

statistically significant. When ANOVA revealed a significant effect, values were compared by Fisher's PLSD post hoc test. All the analysis was conducted with the STATVIEW program (Abacus Concepts, Inc., Berkeley, CA).The percentages of blastocysts at Day 9 pi were based on the total number of cleaved embryos determined at Day 2 pi.

Results

The effects of the bovine embryo different culture methods (microdrop, tube with oil overlay and tube without oil overlay) and serum supplementation on the proportions of the cleaved embryos and blastocysts are shown in Table 3.1. While there were no significant differences ($P > 0.05$) culture methods and serum supplementation interaction and serum supplementation in cleavage and blastocyst rates, means of bovine embryo developmental rate were averaged only in each culture methods for pair wise comparisons (Table 3.2). Regardless of serum supplementation, there were no significant differences ($P > 0.05$) among culture methods in cleavage rates at Day 2 pi (78.3 ± 3.6%, 75.6 ± 2.5%, and 74.9 ± 2.1% for microdrop, tube-oil, and tube methods, respectively). However, the microdrop method was superior ($P < 0.05$) to the tube method without oil overlay for blastocyst formation (30.5 ± 5.4% and 9.8 ± 3.1%, respectively). There was also a trend ($P < 0.10$) of increasing blastocyst rates when embryos were cultured in the microdrop method (30.5 ± 5.4%) compared to the tube method with oil overlay (18.7 ± 3.0). Additionally, the blastocyst rates of the embryos cultured in the tube method with oil overlay (18.7 ± 3.0) tended to be higher ($P < 0.10$) than those cultured in the tube

method without oil overlay (9.8 ± 3.1%). The table 3.1 shows that the supplementation of the culture medium with 10% (v:v) serum at day 5 pi had no significant ($P > 0.05$) effect on the blastocyst rates regardless of the culture methods. Numerically, the highest development rate of blastocysts was observed in the microdrop method with the serum supplementation (36.6 ± 8.5%), and culturing embryos in the tube method without oil overlay and serum supplementation (6.3 ± 3.5%) exhibited the lowest blastocyst rate (Table 3.1).

Table 3.1 Effect of Culture Methods and Serum Supplementation on Bovine Embryo Developmental Rates

Experimental group (culture method-serum supplementation)	Replicates	No. of presumptive zygotes	Cleavage rates (Mean% ± SEM)	Blastocyst rates[4] (Mean% ± SEM)
Microdrop[1]-serum[2]	3	295	78.7 ± 4.9	36.6 ± 8.5
Microdrop-no serum	3	266	77.8 ± 6.2	24.3 ± 5.9
Tube[3] oil-serum	3	286	73.2 ± 2.4	22.2 ± 3.5
Tube oil-no serum	3	273	78.1 ± 4.5	15.2 ± 4.6
Tube-serum	3	280	75.0 ± 0.5	13.2 ± 5.0
Tube-no serum	3	296	74.8 ± 4.7	6.3 ± 3.5
P-value (two-way ANOVA)				
Culture method (Microdrop vs. Tube)			P >0.05	P <0.01
Serum supplementation (serum vs. no serum)			P >0.05	P >0.05
Culture method x serum supplementation			P >0.05	P >0.05

[1]Microdrop method: 50 µl of culture medium in petri dishes.
[2]Serum: 10% (v:v) fetal bovine serum was added at day 5 post insemination.
[3]Tube method: 1 ml of culture medium in test tubes.
[4]Number of total cleaved embryos developing to blastocyst (%).

Table 3.2 Effect of Culture Methods on Bovine Embryo Developmental Rates

Culture methods	Replicates	No. of oocytes	Cleavage rates (Mean% ± SEM)	Blastocyst rates[3] (Mean% ± SEM)
Microdrop[1]	3	561	78.3 ± 3.6	30.5 ± 5.4[a]
Tube[2] oil	3	559	75.6 ± 2.5	18.7 ± 3.0[b]
Tube	3	576	74.9 ± 2.1	9.8 ± 3.1[c]

[1]Microdrop method: 50 µl of culture medium in petri dishes.
[2]Tube method: 1 ml of culture medium in test tubes.
[3] Number of total cleaved embryos developing to blastocyst.
Superscripts differ: [a, b] (P =0.08); [a, c] (P =0.001); and [b, c] (P =0.07).

Discussion

The current study was conducted to evaluate bovine embryo culture conditions as affected by culture methods and serum supplementation. The microdrop and tube methods are generally used among IVF laboratories (Nagy et al., 2003; Rombauts and Wood, 2000). Major differences of these methods are the volume of culture medium and use of oil overlay. The microdrop with oil is the most commonly used embryo culture system because progress of embryo development is easily observed under the microscope and a small volume of culture medium appears to be a beneficial factor for embryo development. Many studies reported that the rates of blastocyst formation and cell numbers were increased when embryos were cultured in the microdrop method where a group of embryo is cultured in a small volume of medium in the mouse (Canseco et al., 1992; Gardner, 2008; Gardner et al., 1994; Salahuddin et al., 1995), sheep (Gardner and Lane, 2004), and bovine (De Oliveira et al., 2005; Keefer et al., 1994; Palasz and Thundathil, 1998). Paria and Dey (1990) interpreted that embryos secrete growth factors that may act in an autocrine or paracrine manner. Thus, the effect of culturing a group of embryos using the microdrop method is likely to be due to the minimization of the diffusion of growth factors secreted from the embryo themselves. The possible evidence of this effect has been demonstrated by culturing bovine embryos in conditioned medium derived from a group of other embryos, which enhances the blastocyst formation rate of single cultured embryos (Fujita et al., 2006). Another assumption is that increasing embryo density (ratio of embryos to volume of culture medium) may deplete embryo-toxic substances in the medium or reduce the concentrations of naturally occurring

inhibitors like glucose or ammonium (Bavister, 1995). Similar to these previous reports, the present results show that regardless of FBS supplementation, the microdrop method was superior to the tube method with the large volume of medium without oil overlay for blastocyst formation (Table 3.2). In addition, there was a trend of increasing blastocyst rates when embryos were cultured in the microdrop method compared to tube method with oil overlay. Fukui *et al*. (1996) similarly reported that the microdrop method resulted in a larger proportion of blastocyst formation than the four-well method, where 500 µl of medium is covered with the oil in the well dish (Fukui et al., 1996). Moreover, their study has shown that the renewal of culture medium at 48 hour intervals in the microdrop lowered the rate of bovine blastocyst formation compared with non-renewal of medium. Thus, the large volume of culture medium and renewal of culture medium causes a decrease in the blastocyst rates presumably by diluting and removing embryo-derived growth factors from the culture (Canseco et al., 1992).

In the present study shown in Table 3.2, the blastocyst rates of the embryos cultured in the large volume of medium with oil overlay tended to be higher than those cultured in the large volume of medium without oil overlay. The primary function of using oil overlay in the embryo culture is that it prevents liquid evaporation which facilitates the maintenance of the appropriate pH and osmotic pressure of culture medium (Boatman, 1987; Brinster, 1968; Gardner and Lane, 2000; Miller et al., 1994b; Tae et al., 2006). Culturing embryos without the oil overlay can lead to low blastocyst formation even though they were cultured in a high humidity environment. Nevertheless, there were no significant differences in the cleavage rate at day 2 pi between the tube method with

and without oil overlay groups (Table 3.2). Thus, the components in the media may have been concentrated during the 9 days of culture resulting in increased osmolarity. Also, Tae *et al.* (2006) have shown that sterilized-good quality oil absorbs the accumulated toxic components in the medium and resulted in an increased rate of development to the blastocyst formation. Nevertheless, other investigators have shown that unidentified toxic components from some particular batches of oil could be transferred into culture medium (Erbach et al., 1995; Miller et al., 1994b; Otsuki et al., 2007; Provo and Herr, 1998), or oil could absorb substances present in the medium such as oestradiol and progesterone (Miller and Pursel, 1987; Shimada et al., 2002). Thus, oil overlay could cause the alteration of medium composition. In this experiment, sterile-filtered, mouse embryo tested, and light mineral oil (M5310; Sigma) was used for the embryo culture, and apparently the mineral oil did not have either significant inhibitory or stimulatory effects on bovine embryo development in the present experiment.

We also investigated the effects of serum supplementation in the culture medium on the developmental ability of the bovine embryos produced *in vitro*. The supplementation of the culture medium with FBS had no significant effect on the blastocyst rates. Serum contains a variety of known and unknown substances which may stimulate or inhibit embryo development *in vitro* (Bavister, 1995; Maurer, 1992). Studies have shown that serum supplementation during the early cleavage stages of culture can inhibit the first cleavage but enhances the formation of morulae and blastocyst when serum is added during the later stage of embryo development (Lim et al., 1994; Pinyopummintr and Bavister, 1991). Many investigators have reported the improved

embryo development with serum-supplemented media (Khurana and Niemann, 2000; Kim et al., 2004; Pinyopummintr and Bavister, 1991; Yoshioka et al., 1997); yet Caro and Trounson (1984) did not find any improvement of the embryo development with the serum-supplemented medium vs. non-supplemented medium in the mouse. Serum is a pathological fluid formed by blood clotting, which may induce chemical alterations with possible harmful effects for embryo culture (Maurer, 1992). It has been reported that some batches of serum decrease the blastocyst rate and cell number and increase apoptotic cells on a particular culture system (Van Langendonckt et al., 1997). Fukui *et al*. (1991) reported that even the effectiveness of serum supplementation in the culture medium varies by different components of culture conditions used *in vitro*. Under these experimental conditions, supplementation with FBS, which was supplemented in either the microdrop or the tube methods, did not statistically improve the rate of blastocyst formation; although embryos derived from culture medium with FBS supplementation exhibited a numerical increase in apparent developmental competence (Table 3.1).

Numerically, the highest blastocyst rates were obtained by the microdrop method with FBS supplementation, and overall, the microdrop method was the optimum culture system among the culture methods (microdrop, tube with oil overlay, tube methods). However, FBS supplementation did not significantly affect bovine embryo development *in vitro*. In the present study, we examined the interactions among culture methods with a constant number of embryos with presence or absence of oil overlay and FBS supplementation in bovine embryo culture. While there are many studies demonstrating positive or negative effects of these components, comparing studies and protocols has

been a difficult process, and it is necessary to examine exact culture methods and batches and lots of serum to be used for *in vitro* culture at the same time as has been described here relative to their impacts on bovine *in vitro* embryo development.

CHAPTER IV

SIMULATED MICROGRAVITY AFFECTS ON BOVINE OOCYTE *IN VITRO* FERTILIZATION AND PREIMPLANTATION EMBRYO DEVELOPMENT

Abstract

The aim of this study was to investigate whether *in vitro* fertilization and preimplantation embryos exposed to a simulated microgravity environment *in vitro* would improve, or be deleterious to, their fertilization and embryonic development. A Rotating Cell Culture System™ (RCCS) bioreactor with a High Aspect Ratio Vessel (HARV) was used to simulate a microgravity environment. *In vitro* fertilization (IVF) and culture (IVC) were conducted in standard microdrop culture method conditions (Control) and simulated microgravity conditions; HARV rotated at 34 rpm (High Speed) and at 3.7 rpm (Low Speed) on a horizontal axis. Embryonic development rates were determined during IVF (Experiment 1), during IVC at presumptive zygote stage (Experiment 2), and IVC at 2-8 cell stages of embryo development (Experiment 3). For IVF studies (Experiment 1), 77.3 % of bovine oocytes were fertilized in the Control group; however, bovine oocytes and sperm fertilization did not occur in High and Low Speed Groups. Moreover, none of the presumptive zygotes (Experiment 2) and 2-8 cell stage embryos (Experiment 3) cultured in High and Low Speed Groups were able to

develop to the further stages. These results indicate that simulated microgravity environments have a negative impact on bovine *in vitro* fertilization and preimplantation embryo development.

Introduction

For several decades, successful space missions have led to growing interests in human space exploration (White and Averner, 2001) and an increasing number of studies have investigated the possibilities of normal animal fertilization and embryo development under microgravity conditions. A variety of animal models have been used, such as medaka fish (Ijiri, 1998), zebra fish (Gillette-Ferguson et al., 2003; Shimada and Moorman, 2006), frog (Souza et al., 1995), salamander (Gualandris-Parisot et al., 2002), mouse (Kojima et al., 2000; Schenker and Forkheim, 1998) and rat (Serova and Denisova, 1982), and studies were done during space flights and in simulated microgravity conditions on the Earth. All of the fertilization and embryo development studies in fish and amphibians showed a similar pattern, that their fertilization and development could occur in space and in simulated microgravity conditions even though there were some degrees of cellular and molecular alterations.

In mammals, only mouse and rat studies have been conducted under microgravity conditions and results have not been consistent. Kojima et al. (2000) reported that simulated microgravity did not affect mouse oocytes and sperm fertilization *in vitro*. However, they also found that preimplantation embryo development in simulated microgravity conditions was significantly decreased compared to a static culture;

although a small portion of 2-cell stage embryos were able to develop to the blastocyst cell stage. In another study, 2-cell mouse embryos were cultured *in vitro* during a flight of the space shuttle Columbia (STS-80). In this experiment, none of the embryos developed to further stages (Schenker and Forkheim, 1998). Moreover in an *in vivo* study, rats were mated in a Cosmos 1129 biosatellite experiment, and although 2 out of 5 females experienced fertilization and early embryo development, the females failed to keep the pregnancies (Serova and Denisova, 1982). These authors speculated that due to the maternal stresses caused by the space flight, the fertilization and embryonic development might be compromised. In their study, one explanation for this embryonic death could be the disruption of embryonic development when the embryo is exposed to microgravity. Thus, mammalian fertilization and early embryo development under microgravity conditions remains unclear requiring more studies to be completed in various mammalian species to further examine the influence of microgravity on developmental processes. In particular, *in vitro* studies will allow us to determine direct effects of microgravity on the embryo itself without effects associated with an altered maternal environment.

In the present study, simulated microgravity culture conditions were obtained using the Rotating Cell Culture System™ (RCCS) bioreactor with High Aspect Ratio Vessel (HARV) (Synthecon, Houston, Texas), which was originally developed by NASA. It has become a useful tool to determine the effects of microgravity on a variety of mammalian *in vitro* cell cultures on the ground. Changes observed in cell morphology and functions in the RCCS bioreactor experiments have proven to be very similar to those

observed after space flights (Goodwin et al., 1993; Ingram et al., 1997; Schwarz et al., 1992; Unsworth and Lelkes, 1998). One major feature associated with the microgravity environment of space is the lack of sedimentation. On the ground, this situation can be created by rotating samples about the horizontal axis in suspension (Hemmersbach et al., 2006; Klaus et al., 1998). The HARV, which is completely filled with medium, is rotated about the horizontal axis at a constant velocity. The combined actions of the centrifugal force, gravity and the viscosity of the medium create the condition of maintaining cells or small samples in suspension (Hammond and Hammond, 2001; Klaus, 2001). Gas exchange is achieved by diffusion through a semi-permeable membrane at the back of the HARV. Additionally, it has been shown that the RCCS bioreactor with HARV generates a low shear simulated microgravity of about 10^{-2} g (Unsworth and Lelkes, 1998). The objective of present study was to determine whether simulated microgravity conditions created by the RCCS bioreactor affect *in vitro* fertilization and preimplantation embryo development using a bovine model system.

Materials and Methods

Oocyte Preparation

All the chemicals utilized in the present investigation were purchased from Sigma Chemicals (St. Louis, MO). Bovine Cumulus-Oocyte Complexes (COCs) were purchased from a commercial supplier (BOMED, INC., Madison, WI). They were shipped in 2 ml

of commercial maturation medium in tightly closed tubes and were over-night shipped in a 39°C portable incubator.

In Vitro Fertilization and Examination of Oocytes

Bovine matured COCs were washed three times in HEPES-TALP (Tyrode's Albumin Lactate Pyruvate; Bioniche, Pullman, WA) wash medium and once in IVF-TALP (Millipore, Billerica, MA) fertilization medium supplemented with pyruvate (0.2 mM), fatty-acid-free Bovine Serum Albumin (BSA-FAF, 6 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), heparin (10 µg/ml), hypotaurine (0.5 µg/ml), and epinephrine (0.5 µg/ml) (Miller et al., 1994a). The matured COCs were placed in groups of 70 in 100 µl of IVF-TALP fertilization medium drops under mineral oil in 60 x 15 mm sterile polystyrene disposable petri dishes (Fisher Scientific, Pittsburgh, PA). For fertilization in simulated microgravity conditions, matured COCs were transferred in microdrops of 100 µl of IVF-TALP covered with mineral oil in 10 ml High Aspect Ratio Vessels (HARV) of the Rotary Cell Culture System™ (Synthecon; Houston, TX). The culture medium and mineral oil were equilibrated at 39°C and with 5% CO_2 in air overnight. All the air bubbles and head space were removed by adding mineral oil in the HARV. All the matured COCs in fertilization medium were placed in a 5% CO_2 incubator at 39°C until spermatozoa were added. The sperm preparation for IVF used the BoviPure™ gradient (Nidacon International AB, Gothenburg, Sweden) and was performed according to the protocol provided by the manufacturer. Briefly, in a 15 ml conical centrifuge tube, 2 ml of BoviPure™ Bottom Layer was placed and then carefully layered with 2 ml of

BoviPure™ Top Layer on the top of the BoviPure™ Bottom Layer to make a gradient. Two 0.5 ml straws of frozen semen from a single Angus bull were thawed in a 37°C water bath for 120 seconds. The thawed semen were gently mixed with BoviPure™ Extender and brought to a 2 ml volume. The 2 ml of the prepared semen was gently layered on top of the BoviPure™ gradient and centrifuged for 20 min at 500 × g. After the centrifugation, the supernatant was carefully removed, and the sperm pellet was resuspended with 5 ml of BoviPure™ Wash and centrifuged for 5 minutes at 300 × g. Then, the pellet was suspended in the IVF-TALP fertilization media. The spermatozoa concentration and motility were then assessed. The spermatozoa were added to the matured oocytes at a final concentration of approximately 2 x 10^6 spermatozoa per 1 ml. Spermatozoa and COCs were co-incubated at 39°C and with 5% CO_2 in humidified air for 18 hours. In order to examine fertilization rates, the presumptive zygotes were denuded from cumulus cells by vortexing for 6 minutes and were fixed in acetic acid and ethanol (1:3/v:v) for 24 hours at room temperature. The presumptive zygotes were mounted on slides with coverslips and stained with 1% aceto-orcein (w:v) for examination by light microscope at a magnification of ×400. Oocytes containing swollen sperm head or pronuclei were considered as fertilized oocytes. Oocytes that were not matured or were degenerated oocytes were not counted.

In Vitro Culture

The cumulus cells of the presumptive zygotes were removed by vortexing for 2 minutes, and the denuded presumptive zygotes were washed 3 times with HEPES-TALP

wash medium. Synthetic Oviductal Fluid (SOF) (Millipore, Billerica, MA) culture medium supplemented with pyruvate (0.4 mM), 100 x MEM (20 µl/ml), 50 x BME (10 µl/ml), BSA-FAF (8 mg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml) was used for culture medium (Edwards et al., 1997). Groups of 70 fertilized oocytes were cultured in 100 µl drops of SOF with mineral oil overlay in 60 x 15 mm sterile polystyrene disposable petri dishes for the static culture condition or 10 ml HARV of the RCCS for the embryo culture in simulated microgravity conditions. Bovine embryos were incubated at 39°C and with 5% CO_2 in humidified air, and the blastocyst rates were assessed at Day 9 post insemination (pi). The day of insemination was defined as Day 0.

Experimental Designs

Experiment 1: Microgravity Simulation during In Vitro Fertilization

The aim of this experiment was to examine the effect of simulated microgravity on bovine *in vitro* fertilization (0 hour to 18 hours pi; see Figure 4.1). Bovine COCs and spermatozoa were co-incubated in the following fertilization conditions. (Control) groups of 70 COCs in 100 µl microdrops of medium with 10 ml of mineral oil overlay in petri dish; (High Speed) groups of 70 COCs in 100 µl microdrops of culture medium with mineral oil overlay in 10 ml of High Aspect Ratio Vessel (HARV) rotated at 34 rpm on a horizontal axis; (Low Speed) groups of 70 COCs in 100 µl drops of culture medium with mineral oil overlay in HARV rotated at 3.7 rpm on a horizontal axis. A schematic representation of experiment groups is shown in Figure 4.2. This experiment was

replicated three times (see Table 4.1 for numbers of oocytes examined), and fertilization rates were assessed at 18 hours pi.

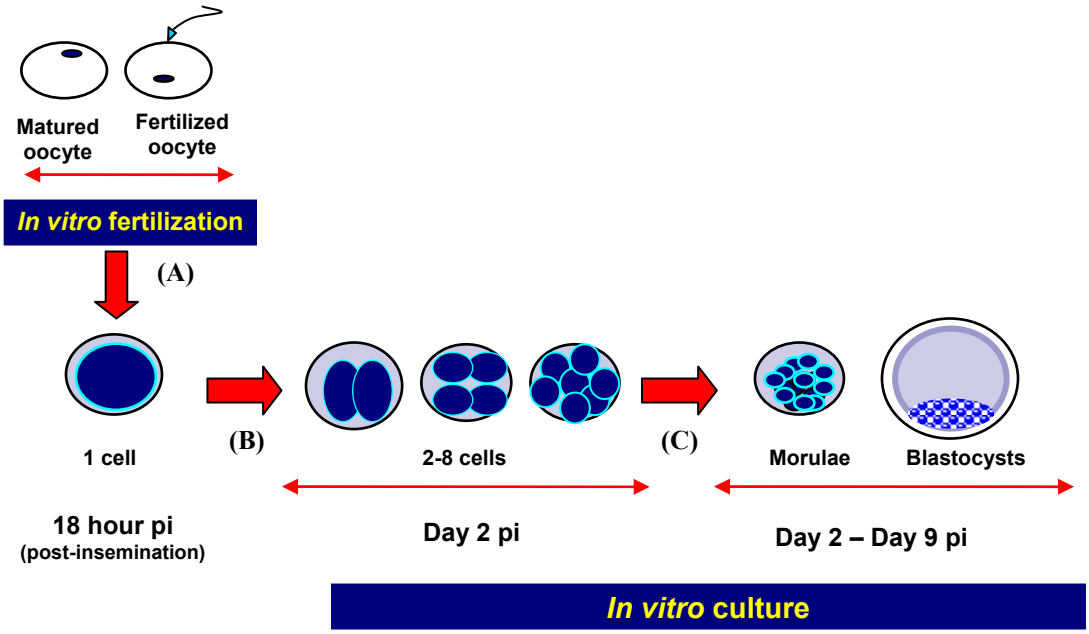

Figure 4.1 Schematic of a Bovine Embryo *In Vitro* Production in Experiment Designs

Note: (A) Experiment 1: microgravity simulation during *in vitro* fertilization (0 hour to 18 hours pi); (B) Experiment 2: microgravity simulation at the 1 cell stage of embryo (from 18 hours to Day 9 pi); (C) Experiment 3: microgravity simulation at the 2, 4, and 8 cell stages of embryos (from Day 2 pi to Day 9 pi).

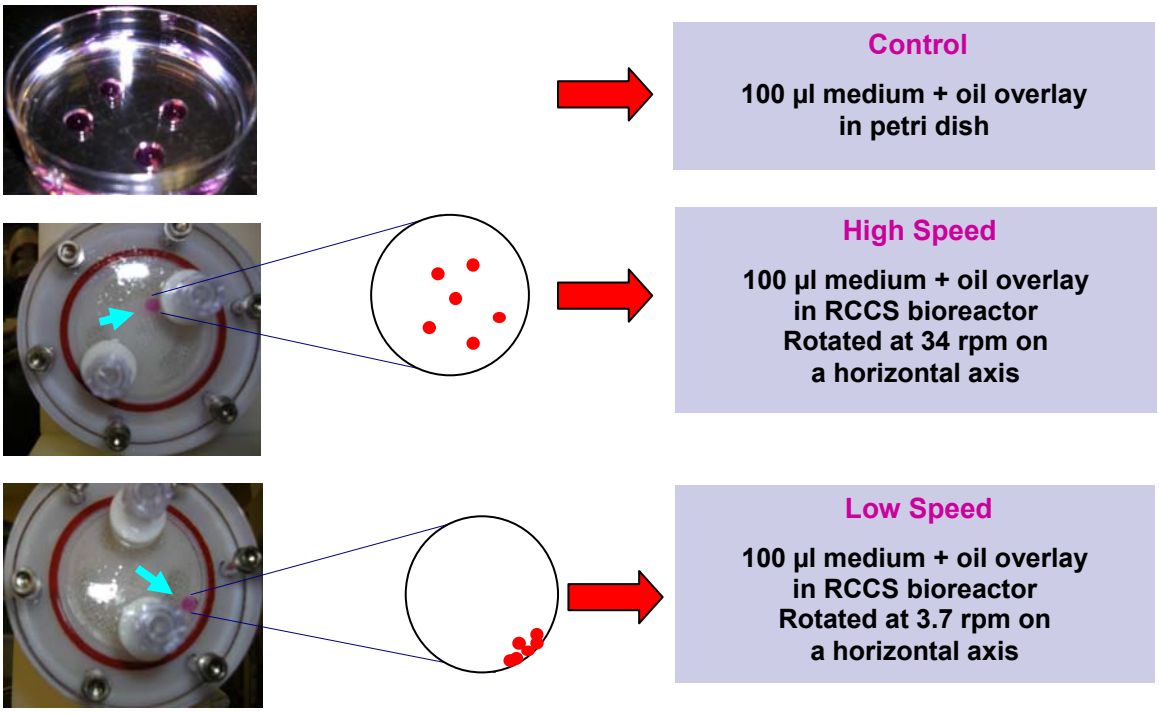

Figure 4.2 Schematic of Experiment Groups (High Speed, Low Speed, and Control Groups)

Table 4.1 Effect of Simulated Microgravity Obtained by RCCS Rotation with 34 rpm (High Speed) and 3.7 rpm (Low Speed) on Bovine Oocyte and Sperm Fertilization.

Treatment Groups	Replicates	No. of oocytes examined	Fertilized oocytes* (Mean % ± SEM)
Control	3	185	77.3 ± 3.7^{a}
High Speed	3	176	0.0 ± 0.0^{b}
Low Speed	3	160	0.0 ± 0.0^{b}

[a, b] Values with different superscripts in the same column are significantly different ($P < 0.05$).
*Oocytes containing swollen sperm head or pronuclei were considered as fertilized oocytes.

Experiment 2: Microgravity Simulation at the 1-Cell Stage

The aim of this experiment was to examine the effect of simulated microgravity on bovine preimplantation embryo development from the presumptive zygotes to the blastocyst stage (from 18 hours pi to Day 9 pi; see Figure 4.1). After IVF, groups of 70 presumptive zygotes were randomly allocated into 100 μl microdrops under mineral oil in a petri dish for the Control group, for the HARV group rotated horizontally at 34 rpm for the High Speed group, and for the HARV group rotated horizontally at 3.7 rpm for the Low Speed group. A schematic representation of experiment groups is shown in Figure 4.2. This experiment was replicated three times (see Table 4.2 for numbers of oocytes examined), and the development rates were assessed at Day 9 pi.

Table 4.2 Effect of Simulated Microgravity Obtained by RCCS Rotation with 34 rpm (High Speed) and 3.7 rpm (Low Speed) on Development of Presumptive Bovine Zygotes.

Experimental group	Replicates	No. of presumptive zygotes cultured	Embryos developing to:* (Mean % ± SEM)	
			Morulae	Blastocyst
Control	3	683	11.0 ± 1.7[a]	3.6 ± 1.3[a]
High Speed	3	601	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Low Speed	3	598	0.0 ± 0.0[b]	0.0 ± 0.0[b]

[a, b] Values with different superscripts in the same column are significantly different ($P < 0.05$).
*Number of total presumptive zygotes developing to morulae and blastocyst at Day 9 pi (%).

Experiment 3: Microgravity Simulation at the 2-, 4-, and 8- Cell Stages (Day 2 pi)

The aim of this experiment was to examine the effect of simulated microgravity on bovine early embryo development from the 2-cell stages to the blastocyst stage (at Day 2-9 pi; see Figure 4.1) of embryos. Following IVF, presumptive zygotes were cultured in 100 µl microdrops of the culture medium covered with mineral oil in a petri dish for 2 days. Cleaved embryos (2-8 cell stage embryos), at Day 2 pi, were equally distributed to Control, High Speed, and Low Speed groups. All the conditions of these three groups were the same as in Experiment 2. All experiments were replicated three times (see Table 4.3 for numbers of oocytes examined), and the development rates were assessed at Day 9 pi.

Table 4.3 Effect of the Simulated Microgravity Obtained by RCCS Rotation with 34 rpm (High Speed) and 3.7 rpm (Low Speed) on 2-8 Cell Stages Bovine Embryos.

Treatment Group	No. of 2-8 cell embryos cultured	Different stages of embryos at day pi (Mean % ± SEM)		
		2-8 cell	> 8-cell	Blastocyst
Control	508	67.7 ± 4.1[a]	15.8 ± 3.1[a]	16.5 ± 2.6[a]
High Speed	508	100.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Low Speed	508	99.6 ± 0.4[b]	0.4 ± 0.4[b]	0.0 ± 0.0[b]

[a, b] Values with different superscripts in the same column are significantly different ($P < 0.05$).

Statistical Analysis

All the experiments were repeated three times and data were analyzed using ANOVA, followed by Fisher's protected least significant difference (PLSD) test using the STATVIEW program (Abacus Concepts, Inc., Berkeley, CA). All percentage data were subjected to arcsine transformation before statistical analysis. Data are expressed as mean ± SEM. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Experiment 1: Microgravity Simulation during *In Vitro* Fertilization

A total of 521 COCs were fertilized with spermatozoa in Control, High Speed, and Low Speed groups and examined for evidence of fertilization after 18 hour pi. As shown in Table 4.1, 77.3 % of bovine oocytes were fertilized in the Control group. However, none of the bovine oocytes in both the High (0.0 %) and Low (0.0 %) Speed

groups were fertilized *in vitro* and no sperm penetration was observed in either of the High or Low Speed groups. Aceto-orcein stained bovine oocytes recovered from the High Speed, Low Speed, and Control Groups at 18 hours pi were shown in Figure 4.3.

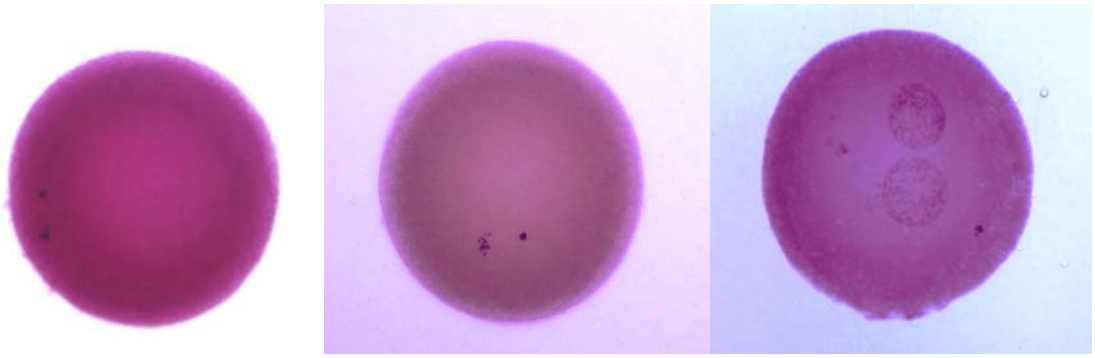

Figure 4.3 Aceto-orcein Stained Bovine Embryos Recovered from the Experimental Groups

Note: (A) High Speed (metaphase II: unfertilized oocyte), (B) Low Speed (metaphase II: unfertilized oocyte), and (c) Control (2-cell stage embryo) groups at 18 hours post insemination.

Experiment 2: Microgravity Simulation at the 1-Cell Stage

A total of 1,882 presumptive zygotes were investigated for their development in simulated microgravity conditions with High (34 rpm) and Low (3.7 rpm) speed rotation. As shown in Table 4.2, while the Control group exhibited 11.0 % and 3.6 % of presumptive zygotes reaching the morulae and blastocyst stages, respectively at Day 9 pi, none of the presumptive zygotes that were cultured in the RCCS bioreactor at High (0.0 %) and Low (0.0 %) Speed groups developed to any other stages of embryo

formation. All the embryos failed to undergo even a single cell division in both the High and Low Speed groups.

Experiment 3: Microgravity Simulation at the 2-, 4-, and 8-Cell Stages (Day 2 pi)

A total of 1,524 2-8 cell stages of embryos were cultured to examine the development of cleaved embryos (2-8 cell stages) from Day 2 pi to Day 9 pi in simulated microgravity conditions with High (34 rpm) and Low (3.7 rpm) speed rotation. As shown in Table 4.3, the proportions of 2- to 8-cell stage embryos in the High Speed and Low Speed groups (100.0 and 99.6%, respectively) were significantly higher ($P < 0.05$) than 2- to 8-cell stage embryos in the Control group (67.7%) on Day 9 pi; with only 1 embryo reaching the morulae stage for the Low Speed group. Thus in the Control group, a greater ($P < 0.05$) proportion of morulae and blastocyst stage (15.8% and 16.5%, respectively) embryos were achieved than in the High (0%) and Low (0% blastocyst and 0.4% morulae) groups. There were no significant differences ($P > 0.05$) in embryo development rates between the High and Low Speed groups with respect to any of the embryo stages noted on Day 9 pi.

Discussion

In the present study, bovine *in vitro* fertilization and preimplantation embryo development have been determined in a simulated microgravity environment. The results have shown that embryonic development was disrupted by the simulated microgravity applied during bovine *in vitro* fertilization and at the presumptive zygotes and 2-8 cell

stages of development. It has been reported that *in vitro* fertilization in the mouse normally occurred in simulated microgravity (Kojima et al., 2000). However, the results from Experiment 1 (Table 4.1) in this study shows that bovine *in vitro* fertilization did not occur in simulated microgravity conditions in either the High or Low Speed groups, and it appears that none of the bovine spermatozoa were able to penetrate the zona pellucida under these conditions. It is usually likely that sperm fail to penetrate the zona pellucida *in vitro* due to decreased motility (Puglisi et al., 2004) or the defective binding of sperm to the zona pellucida (Amann et al., 1999; Braundmeier et al., 2004). The disruption of sperm penetration under simulated microgravity might be related to these two factors. In support of this, human sperm motility has been examined in microgravity environments using the clinostat and parabolic flight models in which sperm motility was found to decrease compared with a 1 g environment (Ikeuchi et al., 2005). Additionally, Andrews and Winters-Hilt (Andrews and Winters-Hilt, 2004) reported that bovine sperm lost their motility within 3 hours under simulated microgravity conditions. Also, they observed abnormal sperm nuclear membranes and mitochondria in sperm under simulated microgravity condition compared to those in a 1 g environment. A review of the literature found no study investigating the effect of microgravity on sperm zona pellucida binding in mammals. However, Tash et al. (2001) reported that the rates of sea urchin sperm binding to oocytes and fertilization were disrupted in a hypergravity environment. Thus, in the present study, the failure of bovine sperm penetration of the oocytes and fertilization under simulated microgravity conditions might be the result of decreased sperm motility and sperm and zona pellucida binding ability. Otherwise, it is

possible that exposure to simulated microgravity for 18 hour during *in vitro* fertilization may lead to bovine sperm and oocyte death since none of the bovine oocytes in the High and Low Speed groups at 18 hours pi showed any sign of fertilization. Additionally, the results of Experiment 2 (Table 4.2) also showed that none of the presumptive zygotes cultured in the RCCS bioreactor for High and Low Speed groups reached any other stages of embryo development. In the development of 2-8 cell stage embryos (Table 4.3), only one embryo reached the morulae stage for the Low Speed group. However, we were unable to exclude the possibility that cell divisions had taken place prior to transfer of embryos from the static culture to the simulated microgravity culture condition. Our results indicated that the simulated microgravity culture conditions were lethal to bovine preimplantation embryos. However, it has been reported that although simulated microgravity can have a negative effect on preimplantation embryo development in mice, 2-cell stage mouse embryos were able to reach blastocyst stages in simulated microgravity conditions obtained by 100 rpm clinostat rotation on a horizontal axis (Kojima et al., 2000). These different results may be explained by either variation of sensitivity toward the microgravity between mouse and bovine species or differing technical aspects of the simulated microgravity. Only one live embryo experiment has been conducted under microgravity conditions of space. *In vitro* culture of 2-cell stage mouse embryos were performed on board the space shuttle Columbia (STS-80) resulting in death of all embryos (Schenker and Forkheim, 1998). The authors concluded that microgravity, as well as possibly other space environment factors, may have caused embryonic death in these mouse embryos.

Because little information is available regarding the effects of microgravity on cellular and functional alterations of spermatozoa, oocytes, and early embryos following exposure to microgravity, it is difficult to explain causes of failed fertilization, development and apparent mortality in the present experiment. However, many *in vitro* culture experiments of various mammalian cells during space flight and under simulated microgravity conditions have shown that microgravity conditions caused decreased cell proliferation, loss of mitochondrial activity and induction of apoptosis (Bucaro et al., 2004; Cogoli et al., 1984; Lewis et al., 1998; Yang et al., 2002). It has been suggested that disruption of the cytoskeleton structure is one of the major causes of cellular changes in microgravity (Lewis, 2004) and is likely to compromise mammalian embryonic development in microgravity as well (Crawford-Young, 2006). Several studies have reported alterations of cytoskeleton structures in response to microgravity in mammalian cell culture *in vitro*. The cytoskeletal structures of papillary thyroid carcinoma cells (Infanger et al., 2006) and glial cells (Uva et al., 2002) in culture were damaged less than 30 minutes after simulated microgravity. In addition, Lewis et al. (Lewis et al., 1998) reported that space flown Human T lymphoblastoid cells exhibited diffuse, shorter microtubules extending from poorly defined microtubule-organizing centers that led to cell death. Thus, the somatic cell death caused by the disruption of cytoskeleton structures described here are likely to occur in sperm, oocytes, and embryos exposed to simulated microgravity *in vitro*; although this was not confirmed as a component of the present investigation. Furthermore, Tabony et al. (2007) showed that self-organized microtubule structures lost ability to transport organelles and chromosomes under brief

periods of simulated microgravity and also proved that the round shapes of cells like embryos were more sensitive to simulated microgravity environments than square shaped cells. The relationship between embryonic development and functions of cytoskeleton under microgravity conditions have not been investigated; however, these somatic cell culture studies under microgravity clearly indicate that disruption of cytoskeleton is probably a major cause of the inhibition of bovine fertilization and embryonic development in the present experiments; although this requires further study to confirm. In conclusion, the present study showed that *in vitro* fertilization and preimplantation embryo development in the bovine was inhibited by simulated microgravity conditions. Further studies are needed to determine the mechanisms through which short term (acute) microgravity conditions can affect *in vitro* fertilization and early embryo development.

CHAPTER V

CONCLUSION

The purposes of this study were (1) to determine the optimum *in vitro* culture conditions for *in vitro* fertilized bovine embryos among different culture methods, and (2) to investigate bovine oocyte fertilization and preimplantation embryo development in simulated microgravity conditions *in vitro*.

In the first part of this study, we determined the interactions among culture methods with a constant number of embryos with or without of oil overlay and fetal bovine serum supplementation in bovine embryo culture. Bovine *in vitro* matured and fertilized embryos were cultured in three different culture methods: microdrop method (50 μl of medium under mineral oil in petri dishes), tube method (1 ml of medium in tube) with oil overlay, and tube method without oil overlay. In addition, the influence of fetal bovine serum in culture methods was examined with in these different culture methods. The results of this study showed that the highest blastocyst rates were obtained by the microdrop method with fetal bovine serum supplementation and, overall, the microdrop method was the optimum culture system among the culture methods. However, addition of fetal bovine serum in the culture medium did not significantly affect bovine embryo development *in vitro*.

In the second part of this study, a Rotating Cell Culture System™ (RCCS) bioreactor with a High Aspect Ratio Vessel (HARV) was used to generate a low shear simulated microgravity conditions. Bovine *in vitro* fertilization and *in vitro* culture of presumptive zygotes and 2-8 cell stage embryos at Day 2 post insemination were conducted in standard microdrop culture method conditions and simulated microgravity conditions: HARV rotated at 34 rpm and at 3.7 rpm on a horizontal axis. The results of this study showed that bovine *in vitro* fertilization did not occur in simulated microgravity conditions. Moreover, none of the presumptive zygotes and 2-8 cell stage embryos were able to develop to further stages, while embryos cultured in the microdrop culture method developed normally. In conclusion, bovine *in vitro* fertilization and preimplantation development were inhibited by simulated microgravity conditions.

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APPENDIX A

BOVINE *IN VITRO* PRODUCTION PROTOCOL

Day -1 (the day of insemination was defined as Day 0)

Preparation of modified TL (Tyrode's albumin Latate pyruvate)-Hepes Wash Medium and IVF (*In Vitro* Fertilization)-TL (Tyrode's albumin Latate pyruvate) solution.

Prepare 44µl drops of IVF-TL and make enough 25µl microdrops for wash incubate them at 39ºC in 5% CO_2 incubator for overnight, and store modified TL-Hepes wash medium in the 4ºC refrigerator.

Table A.1 Modified TL (Tyrode's albumin Latate pyruvate)-Hepes Wash Medium

	Addition	**Concentration**	**Company**	**Cat. #**
TALP-Hepes (ml)	25ml		Bioniche	
BSA, EFAF (mg)	75mg	3mg/ml	Sigma	A-6003
Na Pyruvate solution*	125µl	0.44mM	Sigma	P-4562
Pen/Strep	250µl	100IU/ml	Gibco	15070-063

* Na Pyruvate stock solution (44mM)-dissolve 220mg of Sodium pyruvate in 50ml distilled water. Sterile-filter into an aluminum-foil wrapped bottle and store at 4ºC for 1 month.

Table A.2 Modified IVF (*In Vitro* Fertilization)-TL (Tyrode's albumin Latate pyruvate)

	Addition	**Concentration**	**Company**	**Cat. #**
IVF-TALP (ml)	20ml		Specialty media	BSS-010-D
BSA, EFAF (mg)	120mg	6mg/ml	Sigma	A-6003
Na Pyruvate solution*	100µl	0.44mM	Sigma	P-4562
Pen/Strep	200µl	100IU/ml	Gibco	15070-063

* Na Pyruvate stock solution (44mM) - dissolve 220mg of Sodium pyruvate in 50ml distilled water. Sterile-filter into an aluminum-foil wrapped bottle and store at 4ºC for 1 month.

Day 0

The following procedures are done in the morning (2-3 hours before fertilization).

1. Add 6ml IVF-TALP to a 15ml conical tube. Leave cap loose and prewarm at 39ºC in 5% CO_2.

2. Tighten the caps and place the 25ml of modified TALP-Hepes in 37ºC water bath.

3. Place 2-3 centrifuge carriers in the 37ºC water bath.

Sperm purification using BoviPure™ (Nidacon)

1. A space heater in front of the area where the sperm work will be performed can aid in preventing cold shock to the sperm cells.

2. Make sure that all media used for sperm are warmed at 39ºC before use.

3. Media necessary for fertilization should be prepared at least 2 hours prior to IVF.

4. Use a pipette with a sterile tip to add 2ml of BoviPure Bottom Layer to a conical centrifuge tube.

5. Use new sterile pipette tip to carefully layer 2ml of BoviPure Top Layer on top of the BoviPure Bottom layer.

6. Thaw 2 straws of semen in a 37ºC beaker of warm tap water.

7. Wipe the straws dry with a kimwipe, cut the tip of the straw with a scissors and expel contents of the straw into warm 15ml conical tube.

8. Dilute the thawed semen with Bovipure Extender in 1:1 ratio.

9. Use a sterile Pasteur pipette to carefully layer liquefied semen onto the BoviPure gradient.

10. Centrifuge at 2400rpm for 20 min. Do not use the brake.

11. Use a new sterile Pasteur pipette to aspirate, in a circular movement from the surface, everything except the pellet and 4-6mm of BoviPure Bottom layer. If no pellet is seen after centrifugation, remove all fluid except the lowest 0.5mL.

12. Use a new sterile Pasteur pipette to aspirate the pellet (or the lowest 0.5mL liquid). Transfer sperm pellet to new tube and resuspend pellet in 5ml BoviWash.

13. Centrifuge at 300 x g for 5minutes. Do not use the brake.

14. Aspirate BoviWash supernatant leaving as little liquid as possible above pellet.

15. Resuspend the sperm pellet in a 5ml of warm fertilization media at 1000rpm for 5min.

16. Remove the supernatant and resuspend sperm pellet in 250µl of prewarmed Fertilization media.

17. Mix gently, and keep this original live sperm samples in the incubator.

18. Dilute 10µl sperm to 990µl distilled water and vortex.

19. Add 100µl of diluted sperm to 900µl distilled water and vortex.

20. Load the hemacytometer with diluted sperm.

21. Count sperm cells in 8 large squares on hemacytometer.

22. Number of sperm cells/8=N. N x 10^7 is the number of sperm cells per ml.

23. What we want is a concentration of 2 x 10^6 sperm cells/ml. Because our fertilization media is 50ul, then we need to take 50 x 10^3 sperm cells.

Example: If we have 3 x10^7 sperm cells in 1000ul. Then, 50 x 10^3 sperm cells will be in how many µl (x) X= 5/3=1.6µl.

Preparation of embryo culture drops

1. Prepare embryo culture medium (modified SOF) and 50µl microdrops with mineral oil overlay for culture and make enough 25µl microdrops for embryo wash.

2. Incubate them at 39ºC in 5% CO_2 incubator for overnight to warm up and equilibrate.

Table A.3 Modified SOF (Synthetic Oviductal Fluid)

	Addition	Concentration	Company	Cat. #
SOF	20ml		Specialty media	BSS-046-D
BSA, EFAF (mg)	160mg	8mg/ml	Sigma	A-6003
Na Pyruvate solution*	200µl	0.44mM	Sigma	P-4562
Pen/Strep	200µl	100IU/ml	Gibco	15070-063
BME	200µl	0.5 x	Sigma	B-6766
MEM	400µl	0.5 x	Sigma	M-7145
EDTA	200µl	5µM	Sigma	E-8008

Day 1

Transfer of fertilized oocytes into embryo culture drops

1. After 18-20 hours fertilization, remove cumulus cells from each drops and place in the 500µl microcentrifuge tube. Up to 300 embryos can be loaded in one microcentrifuge tube.

2. Remove cumulus cells from fertilized embryos by vortexing for 2 minutes.

3. Wash the putative zygotes twice with TL-HEPES wash medium.

4. Wash them in SOF wash drops.

5. Allocate the putative zygotes randomly into each treatment.

6. Incubate them at 39 ºC in 5% CO_2 incubator.

Day 2

Preparation of fresh SOF culture media

Assess and record cleavage rate = embryos cleaved/ total embryos placed in microdrops. And, wash embryos once with fresh culture medium and transfer them in fresh 50µl microdroplets.

Day 7- 9

Assess development of embryos to the blastocyst stage.

Reference

Hansen P. J. (2007) Procedures for working with preimplantation embryos. Retrieved from **http://www.animal.ufl.edu/hansen/protocols.shtml** on March 21 2007.

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Samardzija M, Karadjole M, et al. (2006) A comparison of BoviPure and Percoll on bull sperm separation protocols for IVF. Anim Reprod Sci 91, 237-47.

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APPENDIX B

PROGRESS OF BOVINE OOCYTE AND EMBRYO DEVELOPMENT

Aceto orcein satining for bovine embryos

Preparation of 1% aceto-orcein

1. Mix 55ml of distilled water with 45ml of acetic acid (Sigma A9967) to make 45% acetic acid (in hood).
2. Add 500mg of orcein (Sigma O7505) in 50ml of 45% acetic acid
3. Warm orcein acetic acid mixture until orcein is dissolved.

Preparation of 3:1 95% ethanol: acetic acid

1. Mix 5ml of distilled water with 95ml of ethanol to make 95% ethanol.
2. Mix 135ml of 95% ethanol with 45ml of acetic acid

Fix bovine embryos with ethanol acetic acid mixture for 24-48 h.
Stain them with 1% aceto-orcein for 10-20 min.
Observe them under light microscope (Leica DMIRE2) at a magnification of x400.

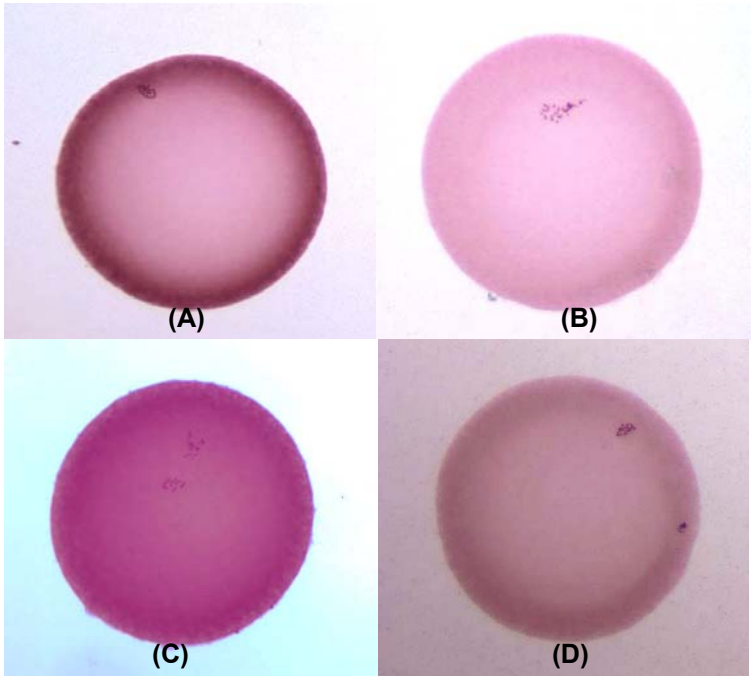

Figure B.1 Aceto-orcein Stained Bovine Oocytes Demonstrating Progress of Nuclear Maturation.

Note: (A) metaphase I, (B) anaphase I, (C) telophase I, and (D) metaphase II.

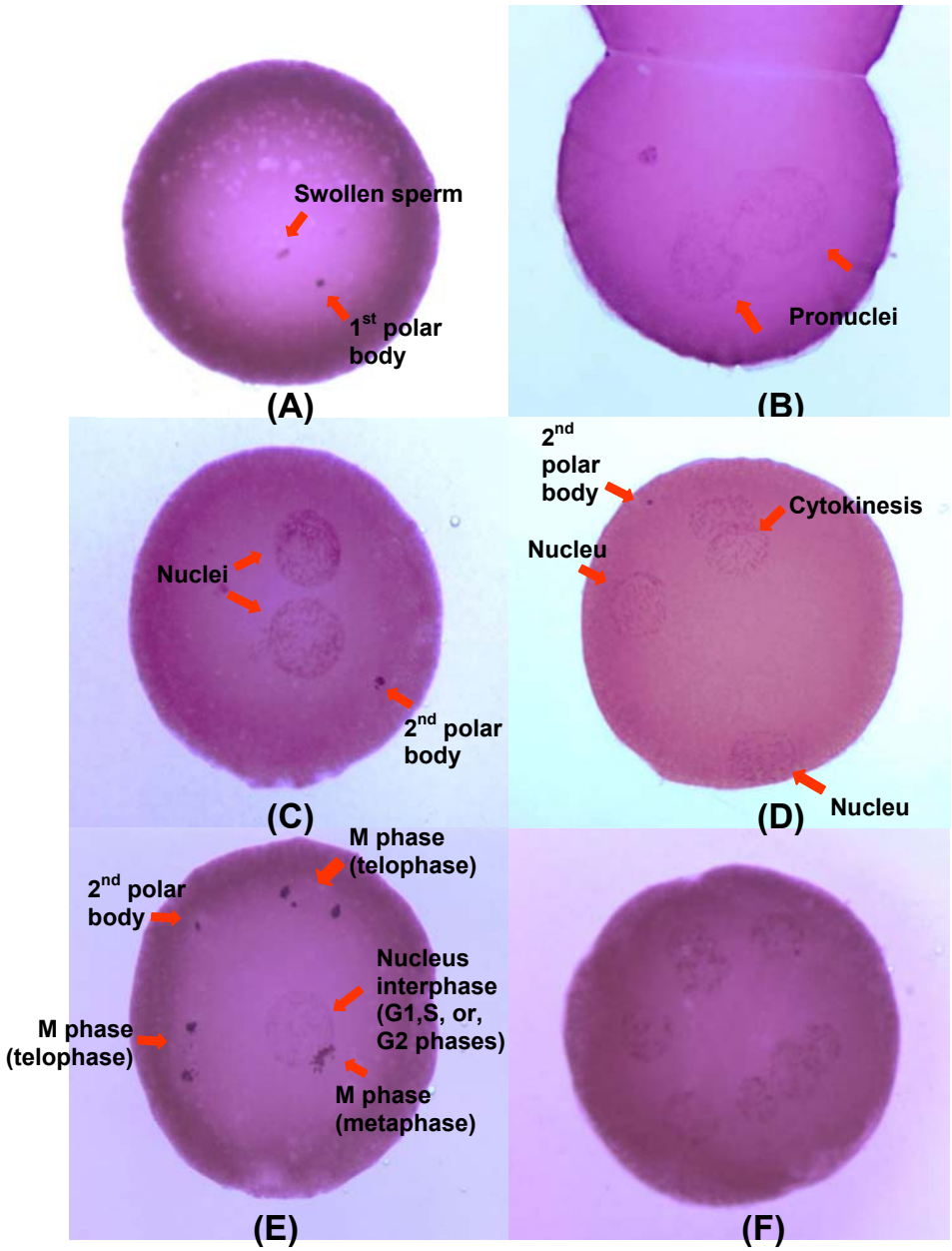

Figure B.2 Aceto-orcein Stained Bovine Preimplantation Embryos Demonstrating Progress of Embryonic Development after In Vitro Fertilization.

Note: (A) sperm penetration, (B) male and female pronuclei formation, (C) 2 cell stage embryo, (D) 4 cell stage embryo, (E) 4 cell stage embryo, and (F) 8 cell stage embryo.