The effect of holding bovine oocytes in follicular fluid on subsequent fertilization and embryonic development

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THE EFFECT OF HOLDING BOVINE OOCYTES IN FOLLICULAR FLUID ON SUBSEQUENT FERTILIZATION AND EMBRYONIC DEVELOPMENT

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program
in Animal Sciences

by
Angela Marie Klumpp
B.S., Louisiana State University, 2001
May 2004
This thesis is dedicated to my Mom and Dad, the two people I could always count on for love and support. I would also like to dedicate this work to the memory of my Nannie, Jerry Simoneaux, the angel who held my hand through this educational journey.
ACKNOWLEDGMENTS

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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bAF</td>
<td>bovine amniotic fluid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>corpora albicantia</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CH</td>
<td>corpora hemorrhagica</td>
</tr>
<tr>
<td>CIDR</td>
<td>controlled internal drug release</td>
</tr>
<tr>
<td>CL</td>
<td>corpora lutea</td>
</tr>
<tr>
<td>E₂</td>
<td>estradiol</td>
</tr>
<tr>
<td>eCG</td>
<td>equine chorionic gonadotropin</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
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<tr>
<td>GVBD</td>
<td>germinal vesicle breakdown</td>
</tr>
<tr>
<td>GV</td>
<td>germinal vesicle</td>
</tr>
<tr>
<td>ICSI</td>
<td>intra cytoplasmic sperm injection</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
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<tr>
<td>IVF</td>
<td>in vitro fertilization</td>
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<tr>
<td>IVM</td>
<td>in vitro maturation</td>
</tr>
<tr>
<td>IVP</td>
<td>in vitro production</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LRS</td>
<td>lactated Ringer’s solution</td>
</tr>
<tr>
<td>M-II</td>
<td>metaphase-II</td>
</tr>
<tr>
<td>M-I</td>
<td>metaphase-I</td>
</tr>
<tr>
<td>P₄</td>
<td>progesterone</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>prostaglandin F₂α</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>TCM-199</td>
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ABSTRACT

The objective of Experiment 1 was to determine the effect of bovine follicular fluid (bFF) on nuclear maturation. Treatment A (Control) oocytes were stained with Hoechst-33342 immediately after aspiration from follicles, whereas, oocytes in Treatment B were held in bFF for 12 hours at 38°C and then stained to determine nuclear status. No significant difference was detected between treatment groups. Results indicate that bFF inhibits resumption of meiosis. The objective of Experiment 2 was to determine the effect of bFF on embryonic development. Oocytes in Treatment A (Control) were placed into in vitro maturation (IVM) for 22 hours followed by in vitro fertilization (IVF). Oocytes in Treatment B were held in bFF for 12 hours at 22°C, followed by IVM and then subjected to IVF. Significantly more (P<0.0001) oocytes cleaved, developed into blastocysts and hatched in Treatment A compared with Treatment B. Results indicate that a 12-hour holding period in bFF does not promote normal embryonic development. The objective of Experiment 3 was to determine the effect of decreased time and concentration of bFF on embryonic development. Treatment A (Control) oocytes were placed into IVM followed by IVF. Oocytes in Treatment B were held in bFF, oocytes in Treatment C were held in Lactated Ringer’s Solution (LRS) and oocytes in Treatment D were held in a combination of bFF and LRS for 6 hours at 22°C, followed by IVM then by IVF. No significant difference was detected between Treatments A and B when analyzing cleavage, blastocyst formation and hatching rates. However, significantly fewer (P<0.0001) embryos reached these stages of development in Treatments C and D. Nevertheless, there were significantly more embryos that
developed to the blastocyst stage in Treatment D compared with Treatment C.

Decreasing the amount of time that oocytes were held in bFF proved to be beneficial in supporting *in vitro* embryo production (IVP). These findings could be advantageous when attempting to rescue valuable gametes from deceased females.
CHAPTER I
INTRODUCTION

The *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of bovine oocytes are valuable tools that can be easily applied for both research and agricultural purposes as described by Gordon (1994). Although modifications to culture conditions used for oocyte maturation, fertilization and embryo development have increased the number of bovine oocytes matured and fertilized *in vitro*, embryo production is still hindered by a high loss during early development. The number of embryos that develop to the blastocyst stage *in vitro* per 100 cumulus oocyte complexes (COCs) is substandard to that for *in vivo* production systems (Sirard and Lambert, 1986). Previous work has demonstrated that ~1 of every 3 oocytes develops into a viable blastocyst following IVM, IVF and IVC (Brackett and Zuelke, 1993). *In vivo* conditions result in higher embryonic development rates, indicating that *in vitro* environments are not optimum for producing live offspring. However, much effort has been devoted to improvement of *in vitro* conditions to allow for efficient bovine embryo production, which could be utilized in both experimental and commercial settings.

The first attempt to use *in vitro* matured bovine oocytes occurred over 25 years ago when fertilization was achieved using a rabbit oviduct to capacitate bull spermatozoa (Iritani and Niwa, 1977). However, suitable culture conditions were later established that allow for IVM, IVF and IVC of bovine embryos resulting in acceptable embryonic growth rates.
The mammalian ovary contains many more immature oocytes than could ever be utilized for reproductive technologies such as IVF and nuclear transfer. Thus, ovarian oocytes are a vast resource of female gametes that could ultimately be harvested with the goal of increasing the numbers of endangered and valuable animals. If oocytes from deceased animals could be rescued, the potential to produce live offspring would benefit not only researchers, but also agricultural producers and zoological researchers attempting to save genetically superior or endangered animals.

However, after the unanticipated death of valuable animals, the resultant postmortem degeneration of oocytes could likely compromise being able to utilize oocytes for animal proliferation. To prevent unnecessary degeneration of oocytes, factors such as time and temperature need to be considered. In cattle, homeostatic mechanisms regulate an environment in which temperatures vary only by tenths of a degree. Temperatures within the developing follicle of cattle are thought to closely correspond with the animal’s body core temperature that is slightly higher than the average rectal temperature of 38°C to 39.3°C (Hewitt, 1921). It has been noted that as temperature at which bovine ovaries are stored decreases, in vitro embryonic development decreases (Yang et al., 1990; Aman and Parks, 1994). Research has also shown that as ovary storage time increases, embryonic development decreases in water buffalo oocytes (Ravindranatha et al., 2003).

In addition to proper ovary storage, other factors such as various holding media and culture conditions of the oocytes need to be taken into account when wanting to rescue genetic material from females. Utilization of follicular fluid as a holding medium
could be beneficial since this is the normal fluid bathing oocytes in an *in vivo* environment. Numerous research studies have analyzed follicular fluid and its subsequent effects on oocyte developmental competence. Follicular fluid has been identified as a transudate of plasma with specific constituents including steroids, glycosaminoglycans, proteins and other metabolites synthesized by the array of follicular cells (Wise, 1987). Follicular fluid undergoes marked alterations in all mammalian species throughout either the estrous or menstrual cycle and regulates various functions including oocyte meiotic inhibition (Tsafriri *et al.*, 1982), cumulus cell expansion and male pronuclear formation *in vitro* (Naito *et al.*, 1988) and it nourishes the oocyte and its adjacent cumulus cells (Edwards, 1974).

Since oocytes naturally acquire developmental competence *in vivo* while being bathed in follicular fluid, it would only be logical to assume this might serve as an alternative *in vitro* holding medium. Several studies have proven the favorable effects of supplementing IVM medium with follicular fluid in the pig (Naito *et al.*, 1988), cow (Kim *et al.*, 1996), sheep (Sun *et al.*, 1994) and horse (Dell’Aquila *et al.*, 1997). However, the stage of the reproductive cycle, size of follicles and concentrations of follicular fluid varied among the previously mentioned studies.

The overall objective of the following studies was to determine whether follicular fluid might serve as a holding or pre-maturation medium in which oocytes could be maintained until they could be utilized under controlled laboratory conditions. If follicular fluid could function as a temporary environment for oocytes, researchers, field zoologists and agricultural producers may some day be able to use this medium to
preserve valuable genetic material for some time without damaging the oocytes. This could ultimately lead to the preservation of genetic material, which could be used to conserve rare, endangered or genetically superior animals.
CHAPTER II
LITERATURE REVIEW

Follicular Fluid

Functions of Follicular Fluid

Follicular fluid contains numerous biochemical components that are essential for ovarian physiology, including steroidogenesis, follicle growth and ultimate maturation of oocytes, ovulation and oviductal transport of the oocyte. In addition, it has been suggested that progesterone in follicular fluid could possibly play a role in the induction of the acrosome reaction in spermatozoa of rabbits (Edwards, 1970), stallions (Cheng et al., 1998; Rathi et al., 2003) and bulls (Lenz et al., 1982). However, follicular fluid does not play a major role in inducing the acrosome reaction in human sperm cells (Mortimer and Camenzind, 1988). Shemesh (1979) concluded that another function of follicular fluid was to inhibit the production of both progesterone and PGF$_{2\alpha}$. Bovine follicular fluid (bFF) from mid-cycle follicles inhibited prostaglandin synthetase as well as luteinization of follicles. However, this inhibition was not noted when follicular fluid from preovulatory follicles was used.

Follicular fluid also functions to provide nourishment to the oocyte and granulosa cells by facilitating transport of specialized nutrients from plasma. The dynamic constituents of this fluid reflect both biochemical and endocrinological activity of the follicle, thereby facilitating its role as a conductor of growth and development through the reproductive cycle (Edwards, 1974). As is the case with any biological fluid, the physical characteristics of follicular fluid are the product of its chemical composition and
reflect its isolation from physiological events occurring outside its environment. In a follicle, physiologic factors are influenced principally by the properties of the tissues, which separate follicular fluid from circulation. The permeability of the follicular wall to water and biochemical components originating from blood can markedly alter the composition of follicular fluid. In addition, the ability of granulosa cells to secrete both stimulatory and inhibitory factors as a result of metabolic processes can modify the composition of follicular fluid. These factors influence physical parameters such as osmolality, color, pH, viscosity and volume of the fluid originating within the developing follicle (Fisch et al., 1990).

It was originally proposed in 1948 that follicular fluid was merely a simple transudate of blood that accumulated between the layers of the granulosa cells in growing follicles (Harter, 1948). It is now known that follicular fluid contains a large variety of components of serum along with an array of secretions, which are synthesized by the specialized cells of the follicular microenvironment. As a result, the components of follicular fluid change during growth and expansion of each follicle (Wise, 1987).

Inorganic Compound, Carbohydrate, Lipid, Vitamin and Protein Content

Follicular fluid is a slightly viscous solution with a pH reported to be 7.4, similar to that of plasma. Investigators have reported various elements of follicular fluid to be similar to those found in serum, with only a few differences (Edwards, 1974; Greve et al., 1989; Luck et al., 2001). For example, in the cow, higher concentrations of potassium and sodium were found in follicular fluid than in serum. However, concentrations of
magnesium, chloride, zinc, copper and inorganic phosphate were similar to those of bovine serum (Petkov et al., 1969).

Biochemical analysis revealed that glucose was ~75% of the total carbohydrate content of bFF with fructose being present as only a minor fraction (Lutwak-Mann, 1954). Lactic acid, cholesterol, protein-bound hexoses and vitamin C were also identified in bFF (Zachariae and Jensen, 1958). Vitamin A has also been identified in bFF, however, its precise function is still unclear. A report by Schweigert and Zucker (1988) suggested that vitamin A may be used as a physiological indicator of follicular quality and function in cattle. The accumulation of vitamin A in follicular fluid has been hypothesized to influence hormone and protein synthesis during follicular development.

An array of proteins found in plasma and follicular fluid has been identified using immunoelectrophoresis and disc-gel electrophoresis (Caravaglios and Cilotti, 1957). The analysis of these proteins has provided evidence that follicular fluid is in partial equilibrium with serum (Edwards, 1974). The majority of plasma proteins are present in bovine follicular fluid although protein concentrations were lower in bFF than in serum (Caravaglios and Cilotti, 1957). For example, $\alpha_2$-globulins were found to be similar in concentration in follicular fluid and plasma whereas albumin and $\beta$-globulin concentrations were lower in follicular fluid than in plasma. Protein concentrations have been reported to be ~75% of that of serum in the cow and 50% in human follicular fluid (Caravaglios and Cilotti, 1957). It has been suggested that the molecular size and shape of the molecules influence their transmission into follicular fluid.
Enzymes

Various enzymes have also been identified in follicular fluid with their concentrations increasing as follicular development progresses. Lactate dehydrogenase, ATPase, transaminases and alkaline phosphatases have been identified in human and bFF (Stallcup, 1970; Caucig et al., 1971). Numerous enzymes found in follicular fluid are involved in the catabolic transformations of compounds that originate in the follicular wall. Specifically, hyaluronidase, endopeptidase and collagenase are three enzymes that exist in follicular fluid to play an important role in ovulation (Zachariae and Jensen, 1958; Rondell, 1970).

Gonadotropins and Steroids

Other important components of follicular fluid are the gonadotropic and steroid hormones. As a follicle grows, the numbers of gonadotropin-binding sites change in both quantity and quality (Channing, 1972). FSH has been shown to play a role in follicular cell proliferation along with the development of the basement membrane. However, FSH and LH together promote the formation of the antrum of each follicle, in addition to helping enable the theca interna cells to become vascularized during development (Eshkol et al., 1970). Steroid hormones, including estrogens, androgens and progestins, have also been identified in follicular fluid. A total of eight steroid hormones were found to be present in bFF, with E2 being present in the highest concentration when follicles reached ~15 mm in diameter (Short, 1962). A key point is that the concentrations of steroids found in follicular fluid vary during different stages of the reproductive cycle.
Concentrations vary widely between different follicles in the same animal or among animals of the same species.

Ohyama et al. (1994) identified two distinct heparin-binding polypeptides in bFF using chromatography. Midkine and pleitrophin concentrations were estimated to be 125 µg/l and 400 µg/l, respectively. These two substances were classified as being heparin-binding growth/differentiation factors, which are believed to play a role in the maturation of ovarian follicles. However, further research is needed to determine the exact function of these two growth factors.

**Ovary Storage**

Under certain circumstances, ovaries can be harvested from the abattoir for research studies. When obtaining ovaries from the abattoir, there are several factors that should not be overlooked. These include the amount of time that ovaries are held at the abattoir before they can be thoroughly cleansed, the transport time between the abattoir and the laboratory and the amount of time it takes before oocytes can be properly harvested from the follicles. Time and temperature are two key factors that can contribute to the detrimental effects on oocyte quality in terms of nuclear maturation and cleavage rates following IVF.

**Influence of Time and Temperature on Oocyte Maturation and Embryo Development**

Solano et al. (1994) found that storing bovine ovaries at 4°C for either 12 or 24 hours had no significant effect on the total number of oocytes that successfully matured and cleaved after fertilization. However, significantly fewer embryos reached the
blastocyst stage, indicating that bovine ovaries could be stored for a short period of time at 4°C and still produce viable blastocysts.

In another study, bovine ovaries were stored at 37°C, 25°C and 4°C for either 0, 4, 8, 16 or 24-hour holding periods (Yang et al., 1990). Cleavage and blastocyst rates were significantly lower for all holding periods when ovaries were stored at 4°C compared with those in the 37°C and 25°C temperature groups. Also, there were no significant differences observed in any of the holding periods for those ovaries stored at 25°C although cleavage and blastocyst yield tended to decrease as holding time increased. Results suggested that bovine ovaries could be stored at 25°C for at least 16 hours without reducing the ability of oocytes to be fertilized and develop to the blastocyst stage. This finding becomes relevant when considering transporting ovaries for research and commercial purposes.

In an experiment conducted with feline oocytes, ovaries were stored in PBS for 24, 48 and 72 hours at 4°C before oocyte recovery. Wolfe and Wildt (1996) concluded that oocytes recovered from ovaries stored at 24, 48 and 72 hours were capable of reaching M-II, indicating that decreased temperature did not affect the meiotic competence of feline oocytes. However, significantly more (P<0.05) blastocysts were produced from those oocytes that were stored in ovaries for only 24 hours compared with those oocytes recovered from ovaries stored for 48 and 72 hours. These results indicated that ovaries stored at 4°C for prolonged periods of time (48 and 72 hours) decreased the oocytes’ developmental competence for subsequent IVF.
In a similar study, feline ovaries were stored in PBS for 16 to 24 hours at 10ºC. Oocyte maturation rates for ovaries stored for 16 to 24 hours and the nonstored controls were not significantly different. Results indicated that decreased temperatures did not negatively affect the meiotic ability of domestic cat oocytes to mature (Katska-Ksiazkiewics et al., 2003) as also shown by Wolfe and Wildt (1996).

Pollard et al. (1996) examined the effect of ambient temperatures during oocyte recovery on the IVP of bovine embryos. Ovaries were collected from the abattoir and transported to the laboratory in PBS at 35°C to 37°C within ~1 to 1.5 hours. Follicles were aspirated in three rooms at which the temperature was maintained at 25°C, 30°C or 35°C, respectively. The total number of oocytes that were penetrated and normally fertilized was not different between the three treatment groups. However, exposing ovaries and cumulus-oocyte complexes (COC) to ambient temperatures below 35°C during oocyte recovery did not decrease the cleavage rate, but it significantly reduced the blastocyst rate. The cleavage rate for oocytes recovered from ovaries at 25°C, 30°C and 35°C was 51.4%, 48.1% and 62.0%, respectively. However, blastocyst rates were significantly higher when the temperature at which oocytes were aspirated increased from 25°C (17.9%) to 30°C (11.2%) or 35°C (28.3%). The authors concluded that ovaries need to be maintained at a temperature no lower than 35°C for oocytes to achieve their maximum developmental potential (Pollard et al., 1996).

Contradictory results were obtained in a study conducted with sheep ovaries (Moodie and Graham, 1991). Storing ovaries for 4, 8 or 24 hours post-slaughter at either 5°C, 22°C or 37°C was found to be detrimental to the percentage of oocytes that matured
in vitro. Ovaries held at 22°C resulted in a greater number of oocytes subsequently maturing compared with those ovaries stored at 5°C and 37°C. These results suggested the optimal temperature to store ovaries until aspiration is near room temperature (~22°C).

Similar findings were found in research conducted with pig ovaries. Rosenkranz (1993) concluded that more oocytes exhibited a greater degree of cumulus cell expansion and obtained higher maturation rates after ovaries had been held at 20°C during transport to the laboratory compared with oocytes collected from ovaries that had been held at 30°C or 37°C. Significantly more oocytes reached M-II when ovaries were transported at 20°C compared with 30°C or 37°C. This finding indicated that increased temperature during ovary transport could elicit detrimental effects on oocyte quality during in vitro studies in the pig.

In a more recent study, Yuge et al. (2003) obtained contradictory results with pig ovaries. Significantly fewer (P<0.05) oocytes were able to reach M-II when ovaries were stored for 1 hour at 4°C, 15°C, 20°C and 25°C compared with those ovaries that were stored at 30°C. These findings concluded that porcine ovaries stored at temperatures lower than 30°C was not appropriate for optimal oocyte maturation.

Influence of Temperature on M-II Oocytes

Research has been conducted to determine the exact effects of cooling oocytes to various temperatures on subsequent competence for IVF. Azambuja et al. (1998) analyzed the effect of low temperature on M-II bovine oocytes. Following IVM, oocytes were maintained at either 39°C (control), 20°C, 10°C or 0°C for 20 minutes after which
IVF was performed. Cleavage rates were significantly higher (P<0.05) for oocytes maintained at 39°C (73.2%) compared with oocytes maintained at 20°C (58.6%), 10°C (47.3%) or 0°C (36.9%). Blastocyst development was also significantly higher (P<0.05) in control oocytes (16.5%) compared with those maintained at 20°C (7.1%), 10°C (0%) or 0°C (0.09%). These data demonstrate that cooling in vitro matured bovine oocytes decreased the percentage of oocytes that undergo fertilization and subsequently develop into blastocysts following IVF.

Liu et al. (2003) also analyzed the effect of cooling on the meiotic spindle of porcine oocytes. Following IVM, oocytes were cooled to either 4°C or 24°C for 5, 30 or 120 minutes. Results indicated that the meiotic spindle in porcine M-II oocytes were sensitive to a drop in temperature (4°C) as indicated by a visible meiotic spindle in only 8% of oocytes compared with the presence of the meiotic spindle in 100% of oocytes held at 24°C. Both the spindle and chromosomes were damaged during the cooling period, and such damage was not reversible after further incubation.

In another study, human oocytes were cooled to room temperature for either a 10-minute or 30-minute time interval. Reduction in meiotic spindle size, disorganization of microtubules within the spindle itself and often times a complete lack of microtubules was observed in all oocytes cooled to room temperature for 30 minutes compared with controls that were maintained at 37°C throughout the experiment. Results indicated that human oocytes were compromised if cooled to room temperature before being subjected to IVF (Pickering et al., 1990).
In an analogous study conducted by Lonergan et al. (1991), bovine oocytes were cooled to room temperature for 30 minutes prior to IVF. Unlike the human, there were no significant differences found between cleavage and blastocyst rates in those oocytes that were held at 37°C compared with those that were cooled to room temperature. It was suggested that bovine oocytes might be slightly more tolerant to decreases in temperature than human oocytes. However, it has been suggested that bovine ovaries should be maintained at no lower than 35°C to promote optimal developmental competence of the oocytes (Pollard et al., 1996).

**Influence of Time Between Slaughter and Oocyte Retrieval**

Results were obtained in mice after studying the effects of the interval between death of the animal and removal of the ovaries on IVF outcome (Schroeder et al., 1991). The total number of cumulus cell-enclosed oocytes decreased as the interval between death and oocyte removal increased from 0 to 12 hours. However, no difference was detected in terms of oocyte maturation. Nevertheless, cleavage rate was lower when ovaries were collected at 9 or 12 hours after the animal’s death and blastocyst rate decreased only after a 12-hour ovary storage period.

An experiment was conducted to determine the effect of time between slaughter and oocyte collection on maturation (Shabpared et al., 1993). Oocytes were collected at 5 to 6, 6 to 7 and 7 to 8 hours following slaughter. Within 1 hour of collection, recovered oocytes from all treatment groups were placed into maturation for 30 hours. Results indicated no differences between treatment groups when comparing cumulus cell expansion and oocyte maturation rates. However, in a more recent report, Love et al.
(2003) found that oocytes stored within ovaries at room temperature for 15 to 18 hours were less capable of maturing (27%) than control oocytes (72%) that were aspirated immediately after slaughter.

Findings with the mare contradict the effects of time between slaughter and oocyte culture on consequent nuclear maturation found in the mouse, sheep, pig and cow (Shabpared et al., 1993). Mare ovaries were held at 30°C to 35°C for either 3 to 9 hours or 10 to 15 hours. Interestingly, there were no differences detected between treatment groups when comparing nuclear maturation rates (Del Campo et al., 1995).

Guignot et al. (1999) also studied the effect of time during transport of equine ovaries on oocyte recovery rate and quality following IVM. Excised mare ovaries were stored for either 1.5 to 4 hours or 6 to 8 hours at 37°C. Significantly fewer oocytes were recovered from ovaries stored for 1.5 to 4 hours compared with those ovaries stored for 6 to 8 hours. Although nuclear maturation was not affected by individual storage times, there tended to be more oocytes with intact cytoplasmic membranes found in those ovaries only stored for 1.5 to 4 hours when compared with ovaries that had been stored for 6 to 8 hours (Guignot et al., 1999).

More recently, Hinrichs et al. (2002) concluded that IVM rates of equine oocytes were significantly increased when oocytes obtained from abattoir-derived ovaries were immediately placed into maturation medium compared with oocytes that were held in tubes containing Earle’s salts and a buffer at 22°C to 25°C while being transported to the laboratory. However, fewer oocytes that were cultured immediately were mature at 24 hours than oocytes held at room temperature. This may be an indication that cytoplasmic
activity can occur in oocytes that are held in medium at room temperature. This may ultimately lead to a faster onset of meiosis after oocytes have been placed into culture (Hinrichs et al., 2002).

Shioya et al. (1988) examined the effect of time between slaughter and aspiration of follicles on the developmental capability of bovine oocytes to mature and fertilize within ligated rabbit oviducts. Ovaries were either aspirated at the abattoir within an average of 17 minutes after slaughter or they were transported to the laboratory in warm saline (38°C) where they were then aspirated an average of 162 minutes post-slaughter. The total number of oocytes that developed to the 2-cell stage after insemination was significantly lower (30.6%) in those ovaries aspirated 162 minutes after slaughter compared with those recovered after only 17 minutes post-slaughter (84.6%). Embryonic development to the 8-cell stage was not different between the treatment groups. However, transfer of embryos to ligated rabbit oviducts resulted in significantly more embryos developing into blastocysts from those oocytes aspirated 17 minutes post-slaughter (28.6%) compared with oocytes aspirated 162 minutes post-slaughter (14.3%). This study suggested that a shorter time between slaughter and aspiration resulted in increased blastocyst production (Shioya et al., 1988), similar to the results reported by Blondin et al. (1997) who found that bovine oocytes collected only 4 hours after slaughter produced significantly more blastocysts (30.5%) compared with ovaries that were aspirated at 6 (13%) and 8 (14.7%) hours following slaughter.

It is always more beneficial for researchers to be able to obtain oocytes from live animals in order to bypass several in vitro obstacles, one of the most important being the
proper storage of ovaries. However, when it is necessary to obtain oocytes from abattoir-derived ovaries, one must keep in mind some of the factors that exert detrimental effects to the oocytes themselves. Previous research has illustrated that time and temperature must be considered when storing ovaries for an extended period of time.

**Ovarian Follicle Size**

**Steroid Concentrations**

When acquiring abattoir-derived ovaries, the exact stage of the reproductive cycle is often unknown. *In vivo* oocyte competence is acquired through hormonal regulation that ultimately causes final maturation of the oocyte and subsequent ovulation. Production of E$_2$ by the somatic cells of ovarian follicles is important in promoting maturation of the oocyte (Kruip, 1985). However, oocyte competence *in vitro* is acquired through the use of complex media, therefore allowing technicians a wide variety of follicles to choose from for the use in assisted reproductive techniques. Consequently, the selection of follicles that have the ability to produce sufficient amounts of E$_2$ is essential for IVM of bovine oocytes. However, the concentration of steroid hormones found in follicular fluid varies with follicle size, degree of atresia and stage of the estrous cycle, thus making follicle selection for *in vitro* studies a difficult task. Oocytes derived from slaughterhouse ovaries constitute heterogeneous material that can lead to decreased blastocyst rates following IVF when compared with those oocytes obtained from animals of known reproductive status (Gordon and Lu, 1990).

A substantial increase in E$_2$ concentration accompanied by a decrease in testosterone is commonly detected with an increase in follicle size. In addition, there is
an increase in IGF-1 as follicle size increased in both porcine and bovine ovaries (Hammond et al., 1985; Spicer et al., 1988). This could be attributed to the fact that IGF-1 functions to regulate follicular cell differentiation (Hazeleger et al., 1995; Austin et al., 2001). In a recent study, Irving-Rodgers et al. (2003) identified insulin-like growth factor binding proteins (IGFBP) in follicular fluid collected from both healthy and atretic bovine follicles. The levels (per volume of follicular fluid) of IGFBP 2, 4 and 5 were not statistically different when analyzing levels of IGFBP between healthy and atretic follicles. It has been hypothesized that the function of IGFBP is to alter the levels of bioavailable IGF that stimulates steroidogenesis and mitogenesis in developing follicles (Bridges et al., 2002). Therefore, an increase in hormone levels in follicular fluid during the estrous cycle may modulate follicular development and/or atresia by affecting the synthesis or proteolysis of IGFBP.

Follicle atresia can be recognized by an increase in P₄ and a decrease in E₂ concentration within bFF (Ireland and Roche, 1982; Kruip, 1985). Dominant follicles have been shown to have high E₂ and low P₄ levels. The variation in the concentration of these steroids in follicular fluid during various stages of the cycle indicates the ability of follicular cells to change hormone production (Dobson and Dean, 1974; Wise et al., 1982; McNatty et al., 1984). Altering hormone output of the follicular cells has an effect on an oocyte’s potential to develop.

The influence of follicular size and stage of the cycle on steroid hormone concentration in follicular fluid can be influenced by the difference in availability of LH and FSH receptors and by the variation in LH patterns (Karsch, 1977; Roche and Ireland,
1981; Hansel, 1983). Although FSH primarily influences the system for androgen aromatization, LH receptors are also found on the granulosa-cell membrane. Both FSH and LH have proven to be beneficial for the large production of E₂ within the follicle (England et al., 1981; Hillier, 1981; Merz et al., 1981). Bovine follicles ranging in diameter from 2 to 5 mm have LH receptors located in the theca interna and will produce androgens; however, these small follicles have very few LH receptors on the granulosa-cell membrane. These small follicles have been termed “androgen-dominated” as a result of the lack of LH receptors that will ultimately aromatize androgens into estrogens (Tsonis et al., 1984). Follicles >11 mm in diameter have been classified as “estrogen-dominated”. As these follicles grow, there is an increase in LH receptors in both the theca and granulosa cells, which contribute to the increased output of E₂.

The differences in steroid concentrations between follicles of various sizes can be attributed to intrinsic factors, such as the capacity to produce steroids, and extrinsic factors, such as the availability of gonadotropic hormones. Therefore, a key aspect contributing to developmental competence of oocytes is the concentration of various hormones in follicular fluid (Sirard, 2001).

Factors Affecting Oocyte Developmental Competence

There are several ways to determine the developmental competency of an oocyte: polar body extrusion, ICSI, cleavage rates, blastocyst formation and cumulus cell expansion. In addition, staining oocytes to determine their stage of nuclear maturation can also be used to determine an oocyte’s ability to become fertilized and ultimately develop into a viable embryo in vitro (Carnevale et al., in press).
Studies have shown that the developmental capacity of bovine oocytes is affected by several factors, two of which include the size (Tan and Lu, 1990; Pavlok et al., 1992; Carolan et al., 1996) and quality of the follicle (Blondin and Sirard, 1994; Blondin et al., 1997; Fouladi Nashta et al., 1998). These factors were considered in a study that investigated the capacity of bovine oocytes derived from follicles of different sizes to undergo normal fertilization and early embryonic development in vitro (Pavlok et al., 1992). Follicles were categorized into three groups: >4 to 8 mm (large), >2 to 4 mm (medium) and >1 to 2 mm in diameter (small). After IVF, embryonic development was recorded and it was noted that there were significantly more embryos that cleaved from oocytes recovered from large follicles (68.9%) compared with those recovered from medium (64.3%) or small (19.7%) follicles. Similarly, blastocyst development followed the same pattern. Significantly more embryos developed to the blastocyst stage after oocytes had been recovered from large follicles (27.7%) than from medium (20.9%) or small (0%) ones. These results demonstrate the correlation between follicular size and oocyte developmental competence. It is apparent that oocytes obtained from small bovine follicles do not have the same capacity to develop in vitro as do oocytes acquired from larger follicles.

Lonergan et al. (1994) also investigated the effect of the size of bovine follicles from which the oocytes originate on their subsequent in vitro developmental ability. Follicles were categorized into two groups based on diameter: 2 to 6 mm (small) and >6 mm (large). Cleavage rates were not different between the small (92.9%) and large (90.8%) follicle groups. However, blastocyst development was significantly lower for
those oocytes retrieved from small follicles (34.3%) compared with oocytes recovered from large follicles (65.9%).

In a study conducted on tamer wallaby oocytes, Mate and Rodgers (1993) studied the effect of follicle size on oocyte meiotic competence. Oocytes obtained from small (<1.5 mm) and large (≥1.5 mm) follicles were placed into maturation medium. Significantly more oocytes reached M-II from large follicles (55%) compared with oocytes recovered from small follicles (20%). This study clearly illustrated that a relationship between follicle size and oocyte quality in terms of in vitro developmental and meiotic capacity does exist, with oocytes originating from larger follicles being superior to those originating from smaller tamer wallaby follicles.

Another factor, in addition to follicle size and quality, that affect IVM are serum type, hormones and the addition of cumulus cells (Fukui et al., 1997). Oocyte maturation rates in the Minke whale significantly increased from 6.6% when oocytes were exposed to only TCM-199 and fetal whale serum (FWS) to 21.6% when oocytes were exposed to TCM-199 supplemented with fetal whale serum (FWS), LH, FSH and cumulus cells.

**Oocyte Diameter**

Another factor that can be correlated with follicular size and the outcome of IVF is oocyte diameter. The oocyte growth phase includes a series of modifications of cell organelles. These modifications are crucial for an oocyte to attain meiotic and developmental competence. Fair et al. (1995) identified a possible relationship between the diameter of bovine oocytes and follicle size and RNA transcriptional activity. Ovarian follicles were classified into five groups based on size: ≥4 mm, >3 to <4 mm, >2
to 3 mm, >1 to 2 mm and <1 mm. A positive correlation was found between oocyte diameter and follicle size. It was noted that bovine oocytes achieve maximum size when the follicle has developed to 3 mm and after that oocyte growth levels off. Hendriksen et al. (2000) also documented that oocytes acquire the capacity to develop into an embryo following IVF at the follicular stage of 3 mm. However, they require an additional “pre-maturation” to express this competence. Pre-maturation of oocytes occurs in vivo prior to the LH surge whereas, in vitro, oocytes are exposed to medium which aids in the oocyte’s ability to mature, to be fertilized and to develop into a viable embryo (Hendriksen et al., 2000). Oocyte diameter varied from 99 µm for oocytes recovered from antral follicles <1 mm to 117 µm for oocytes from follicles reaching >4 mm in diameter. Similar findings in relation to oocyte diameter were reported by Suzuki et al. (1994). However, slightly different results were obtained from a study conducted by Motlik and Fulka (1986). They found that the mean oocyte diameter was ~110 to 113 µm for porcine oocytes from follicles <1 mm in size.

The ability of bovine oocytes of different sizes to mature in vitro has also been investigated (Fair et al., 1995). Oocytes were placed into four treatment groups based on diameter: oocytes <100 µm, 100 to 110 µm, 110 to 120 µm and >120 µm in diameter. Following IVM, a significantly larger number of oocytes were at M-II when oocyte diameter was 110 to 120 µm and >120 µm when compared with oocytes having a diameter of <100 µm and 100 to 110 µm. More oocytes with a diameter of <100 µm remained at the germinal vesicle (GV) stage of nuclear maturation. It appeared that bovine oocytes acquire the ability to complete nuclear maturation at a diameter of...
~110 µm. This report is comparable to the results obtained by Fuhrer et al. (1989), Hyttel et al. (1997) and Sirard et al. (1998), all of which concluded that oocytes gradually acquire competence to undergo meiotic maturation and sustain embryonic development after reaching a diameter between 110 and 120 µm. The degree of RNA synthesis was determined by culturing bovine oocytes in the presence of 3H-uridine for 45 minutes. Autoradiographic labeling showed a greater degree of RNA synthesis in oocytes <100 µm and 100 to 110 µm in diameter when compared with oocytes ranging from 110 to 120 µm and > 120 µm. These results indicated that oocytes of smaller diameter were involved in RNA synthesis and were consequently still in the growth phase (Fair et al., 1995).

Although bovine oocytes obtained from small follicles normally do not acquire competence until later in follicular growth, Mermillod et al. (2000) found a pre-maturation culture system that could be applied to these oocytes in vitro to reproduce the last steps of oocyte differentiation that normally occur late in folliculogenesis.

Roscovitine is a known inhibitor of maturation promoting factor (MPF) kinase activity in bovine oocytes. The addition of 25 µM of roscovitine to the culture medium was found to maintain bovine oocytes at the GV stage for a 24-hour period. The inhibitory effects of this substance were also found to be reversible after exposing oocytes to TCM-199 and 10 ng/ml of EGF. Approximately 90% of the oocytes reached the M-II stage and further developed to the blastocyst stage after being exposed to roscovitine and EGF compared with control oocytes. These findings may allow for the setup of a culture system
specially designed for oocytes known to have low developmental competence, especially those recovered from follicles smaller than 3 mm in diameter (Mermillod et al., 2000).

**Oocyte Maturation**

Maturation of oocytes can be divided into two different stages, nuclear maturation and cytoplasmic maturation. Nuclear maturation can be defined as the series of physiologic events that reflect the modification of chromatin from the diplotene phase (GV) to M-II. Cytoplasmic maturation encompasses all of the changes in the distribution and organization of specific organelles from the GV to the M-II stage. Cytoplasmic maturation is associated with the cellular changes that occur during nuclear maturation such as mitochondria repositioning and cortical granule migration (Sirard, 2001). Various hormonal and follicular aspects reported to enhance both nuclear and cytoplasmic maturation in bovine oocytes are shown in Figure 2.1. See Sirard and Blondin (1996) for a review of oocyte maturation and IVF in cattle.

**Meiotic Arrest**

It has been estimated that there are >150,000 oocytes in the ovaries of a heifer at birth. The process of meiosis begins early in fetal life but is arrested shortly after birth at the diplotene stage. Oocytes will either degenerate through the process of atresia or resume meiosis just prior to ovulation. Normal meiosis is resumed in the adult following the preovulatory surge of gonadotropins (Ayalon et al., 1972; Vermeiden and Zeilmaker, 1974; Tsafiriri et al., 1976).

Since 1935, researchers began to investigate the spontaneous events that lead to oocyte maturation. Pincus and Enzmann (1935) were the first to report the spontaneous
Figure 2.1. Overview of the key factors that regulate nuclear and cytoplasmic maturation in oocytes (Hunter, 1998).
nature of nuclear maturation by mammalian oocytes recovered from antral follicles. They hypothesized that follicular components supplied the oocyte with a substance that directly inhibited nuclear maturation (Pincus and Enzmann, 1935). The phenomenon of spontaneous maturation was later reported in mouse, sheep, cow, pig, rhesus monkey and human oocytes (Edwards, 1965). Two mechanisms were suggested to affect meiotic arrest: (1) follicular fluid was mediating the inhibitory action on meiotic resumption (Jagiello et al., 1977; Meinecke and Meinecke-Tillmann, 1981) or (2) close contact of the oocyte to follicular cells was needed for an inhibitory signal to be transferred (Colonna et al., 1989; Racowsky and Baldwin, 1989).

Several researchers have observed gap junctions between cumulus cells and the oocyte (Unwin and Zampigni, 1980; Loewenstein, 1987; Sutovsky et al., 1993). Physiologically, the function of follicular gap junctions is to provide nutrition to and from cells, electric coupling and the transport of messenger molecules from follicular cells to the oocyte (Lawrence et al., 1978). Transfer of ions and small molecules between the oocyte and cumulus cells were found to be maximized just prior to gonadotropin stimulation and decreased thereafter (Moor et al., 1980). This finding led researchers to believe that there was a disassociation of the oocyte-cumulus cell gap junctions that would ultimately lead to meiotic resumption in oocytes, possibly induced by the gonadotropin surge prior to ovulation (Linder et al., 1974). The transmission of inhibitory substances was thought to be involved with the gap junctions that connect the cumulus cells to the oocyte (Albertini and Anderson, 1974; Gilula et al., 1978; Larsen and Wert, 1988; Motta et al., 1994).
Sirard and Bilodeau (1990) determined that granulosa cells produced a factor that inhibited nuclear maturation in bovine oocytes cultured in vitro. Other authors have suggested that there is an LH-induced loss of gap junctions that triggers the resumption of oocyte maturation by reducing the transport of meiosis inhibitory substances to the germinal compartment of the follicle (Larsen et al., 1986; Racowsky and Baldwin, 1989; Wert and Larson, 1990). These findings led to the hypothesis that follicular somatic cells inhibit nuclear maturation.

An inhibitory effect of follicular fluid on the spontaneous maturation of oocytes was first described by Chang (1955) in the rabbit. It was later found that follicular fluid inhibited maturation of pig (Tsafiriri et al., 1976), rat (Tsafiriri et al., 1977, 1978), hamster (Gwatkin and Andersen, 1976), sheep and human oocytes in vitro. When the medium was supplemented with LH, oocytes were able to resume meiosis. This finding confirmed the role of LH in the control of the resumption of meiosis in vivo.

Similarly, in the cow, follicular fluid decreased the percentage of oocytes that were able to mature (Sirard et al., 1992). Romero-Arredondo and Seidel (1994) concluded that bFF collected at 0 and 4 hours following the LH surge inhibited maturation of oocytes. However, this inhibitory effect was absent in bFF collected at ≥8 hours after the LH surge, as indicated by increased cumulus expansion and meiotic maturation compared with control oocytes.

Secretion of Inhibitory Substances

Other studies have identified several substances in follicular fluid that prevented the resumption of meiosis in the oocyte. Mullerian inhibiting substance, a by-product of
granulosa cells, has been found to inhibit maturation of rat oocytes (Takahashi et al., 1986). Linoleic acid (18:2), the most abundant fatty acid found in follicular fluid, was found to inhibit meiotic resumption of denuded bovine oocytes (Homa and Brown, 1992). Furthermore, another substance, termed oocyte maturation inhibitor (OMI), has been reported to suppress the maturation of rat and pig oocytes (Tsafriri et al., 1982). The original source of OMI was thought to be the granulosa cells; however, it was found that both the theca interna and granulosa cells interacted to play a key role in maintaining meiotic arrest in the bovine oocyte (Richard and Sirard, 1996a, 1996b). Hillensjo et al. (1979) found OMI inhibited the resumption of meiosis only in those oocytes cultured within their intact cumuli, but failed to affect the maturation of denuded pig oocytes. This indicated that the inhibitory signal of OMI is communicated to the oocyte through the interactions of the cumulus cells (Tsafriri et al., 1982).

Hypoxanthine, a purine commonly found in follicular fluid, has been shown to exert negative effects on oocyte maturation. In the mouse, hypoxanthine has been reported to arrest oocytes for a prolonged period of time (Downs et al., 1985, 1989). Kadam and Koide (1990) found that the concentration of hypoxanthine in bFF was able to inhibit maturation of mouse oocytes in vitro. They hypothesized that hypoxanthine may not be the only substance in follicular fluid that maintained meiotic arrest in mouse oocytes, but rather one of several factors.

Inhibin, a glycoprotein hormone, is synthesized by the granulosa cells of ovarian follicles and accumulates in the follicular fluid (Erickson and Hssueh, 1978; Henderson and Franchimont, 1983). The major function of inhibin is to suppress the release of FSH,
thereby negatively affecting follicular growth. W.-S. et al. (1989) reported that rat oocytes were prevented from maturing when exposed to inhibin. In addition to OMI, hypoxanthine and inhibin, 20β-dihydroxyprogesterone was also identified as being a meiotic inhibitor of mouse oocytes (Barrett and Powers, 1993).

Although there may be numerous factors involved in oocyte maturation, it is believed that the most prominent feature of the agents inducing oocyte maturation in vitro is their ability to stimulate the production of cAMP (Sirard, 1990). The cAMP pathway involves the activation of protein kinase A, which phosphorylates proteins within the oocyte that maintain meiotic arrest (Schultz, 1988). Protein kinase A is part of a signal transduction pathway that utilizes adenylate cyclase to convert ATP into cAMP. This process is under control of G-proteins (Mochly-Rosen, 1995).

When mouse and rat oocytes were cultured in the presence of cAMP derivatives, spontaneous maturation was prevented (Cho et al., 1974; Nekola and Moore-Smith, 1975; Wassarman et al., 1976; Dekel and Beers, 1978; Downs and Eppig, 1984). Similarly, maturation of amphibian oocytes is induced by an increase in P₄ along with a decrease in cAMP (Morrill et al., 1977; Speaker and Butcher, 1977; Schorderet-Slatkine et al., 1978; Maller et al., 1979). In contrast, cAMP increases in sheep oocytes before resumption of meiosis (Moor et al., 1981). However, it has been suggested that cAMP may serve as the physiological inhibitor of meiosis, which is transmitted from the cumulus cells of the follicle through distinct gap junctions.

Consequently, cAMP has been proposed to be the inhibitory signal that maintains bovine oocytes in the GV stage of nuclear maturation (Eppig and Downs, 1984; Homa,
A decrease in cAMP levels within the oocyte appears to be the initial step in the cascade of events ensuring oocyte maturation (Aktas et al., 1995). Other substances, such as hypoxanthine and adenosine act synergistically to help maintain increased cAMP concentrations, thus promoting meiotic arrest.

To further prove the inhibitory role of cAMP in resumption of meiosis, researchers have studied the effects of certain chemicals that elevate intracellular levels of cAMP such as dibutyryl cAMP, forskolin, cholera toxin, sodium fluoride, prostaglandin E₂, 6-dimethylaminopurine and cycloheximide. Increased levels of these chemicals ultimately leads to inhibition of nuclear maturation in bovine oocytes due to their ability to increase cAMP levels within the oocyte (Sirard and First, 1988; Sirard, 1990; Bilodeau et al., 1993; Aktas et al., 1995).

Resumption of Meiosis

Resumption of meiosis in bovine oocytes (see Sirard et al., 1998 for review) requires the synthesis of certain proteins at the onset of maturation (Simon et al., 1989; Sirard et al., 1989; Tatemoto and Terada, 1995). During the first hours of culture, proteins that aid in nuclear envelope breakdown are synthesized. Thereafter, bovine oocytes continue to require protein synthesis throughout the entire process of maturation (Kastrop et al., 1991; Milovanov and Sirard, 1994). Protein synthesis of the oocyte to resume meiosis has been correlated to the amplification of MPF, along with the synthesis of cyclin B, an integral component of MPF activity.
Downs (1993) reviewed the factors affecting resumption of meiosis in mammalian oocytes. There have been two proposed hypotheses for the mechanism involved in meiotic maturation: (1) a loss of inhibitory input and (2) positive stimulation. After the preovulatory gonadotropin surge, gap junctions become disrupted, allowing for a loss of communication between follicular cells and the oocyte. This ultimately interrupts the flow of inhibitory substances such as cAMP to the oocyte and indirectly promotes the onset of maturation (Dekel et al., 1988; De Loos et al., 1994; Hyttel, 1997). The second hypothesis suggests there is a positive factor that acts on the oocyte to override the inhibitory influence, thereby inducing the onset of germinal vesicle breakdown. Calcium has been identified as a possible candidate due to research reporting spontaneous maturation of oocytes following exposure to calcium ionophore, A23187 (Powers and Paleos, 1982; Racowsky, 1986).

When resumption of meiosis finally occurs, it is regulated by a considerable increase in the cytosolic kinase activity of the oocyte. Researchers have shown that MPF, comprised of the p34⁰cdc² kinase and cyclin B, is a crucial component of this activity (Dekel, 1996; Levesque and Sirard, 1996; Whitaker, 1996). Downs (1995) identified MPF as a serine-threonine protein kinase that is involved in regulation of the cell cycle. It is also known that MPF increases after germinal vesicle breakdown, with a decline in MPF when the first polar body is extruded.

Effects of Follicular Fluid

Given that resumption of meiosis and cytoplasmic maturation of oocytes takes place in close association with follicular fluid, supplementing media with follicular fluid
would be a logical choice for researchers. Many researchers have looked at the effects of supplementing maturation media with follicular fluid on subsequent maturation and embryonic development. Chauhan et al. (1997) replaced serum and hormone additives with follicular fluid in the IVM medium to look at the effects on maturation, fertilization and embryonic development of buffalo oocytes. Follicular fluid was collected from all visible follicles and then pooled. Nuclear maturation rates were similar between oocytes placed into TCM-199 + 10% FBS + 5 µg/ml pFSH (74%), TCM-199 + 20% follicular fluid (FF) (67%) and TCM-199 + 40% FF (67%), but these were significantly higher than those oocytes matured in TCM-199 + 10% FBS (47%). There was also a greater degree of cumulus expansion in those oocytes matured with 20% or 40% FF although cleavage, morulae and blastocyst rates did not differ. Results from this study suggested that buffalo follicular fluid had substances present, which promoted both nuclear and cytoplasmic maturation, therefore indicating its potential as a possible supplement for IVM medium.

Comparable results were obtained when follicular fluid from large bovine follicles (≥15 mm) was added to maturation medium (Elmileik et al., 1995). Follicular fluid had no direct stimulatory effects on the rate of nuclear maturation. However, the addition of 10% to 15% bFF to the maturation medium resulted in significantly higher rates of morula, blastocysts and hatched blastocysts compared with the controls. The authors concluded that follicular fluid from follicles ≥15 mm in diameter contained stimulatory agents that during IVM promoted the developmental potential of the oocytes to develop into viable embryos.
Similar results were obtained by Kim et al. (1996) when using follicular fluid collected from small follicles (<5 mm) as a supplement to the maturation medium. Medium supplemented with 10% or 30% FF did not affect the oocytes development to the blastocyst stage, whereas 60% FF significantly decreased embryonic development. Also, there were no differences detected when analyzing the total number of cells present from day-9 blastocysts. These results suggest that by supplementing bFF from small follicles to IVM medium, one would be able to promote normal embryonic development (Kim et al., 1996).

Romero-Arredondo and Seidel (1996) also noted normal embryonic development followed by maturing bovine oocytes in 10% or 40% FF for 20 hours. A slightly different trend was noticed by Choi et al. (1998) when they compared IVM rates of bovine oocytes matured in medium supplemented with follicular fluid collected from large (10 to 20 mm) or small (2 to 5 mm) follicles. Maturation medium consisted of TCM-199 supplemented with 1 IU ml⁻¹ of eCG and follicular fluid from either large or small follicles. It should be noted that maturation was conducted in co-culture with bovine oviductal epithelial cells.

Significantly more oocytes matured in the medium supplemented with bFF from small (77.9%) compared with large follicles (69%). However, significantly more oocytes underwent maturation (91.7%) when no bFF was added to the maturation medium. No differences were noted with the total number of oocytes that became fertilized between treatments, although bFF from large follicles decreased the rate of male pronucleus formation. There were a greater number of embryos that developed to the blastocyst
stage when bFF from small follicles (15.4%) was utilized compared with bFF from the large follicles (5.3%). However, blastocyst rates were significantly lower from those oocytes matured in follicular fluid recovered from both large and small follicles when compared with the controls (31.8%). These findings indicate that the developmental capacity of bovine oocytes to mature in follicular fluid is dependent on the developmental stage of the follicles from which the fluid was collected (Choi et al., 1998).

Although contradictory results were previously reported by Elmileik et al. (1995), the total concentration of FF added to the medium could have played a factor with the deceased rates observed by Choi et al. (1998). Decreased maturation rates were also noted by Ayoub and Hunter (1993) when bovine follicular fluid was added to the maturation medium. Follicular fluid collected from small follicles (2 to 4 mm) was only able to promote nuclear maturation in 1.4% of the total number of oocytes. Follicular fluid from medium-sized follicles (5 to 9 mm) promoted maturation in 1.3% of oocytes whereas, fluid collected from large follicles led to maturation of 2.7% of oocytes cultured. These results were significantly lower than in medium containing only TCM-199 (46.2%). Therefore, bFF aspirated from small, medium and large follicles was able to inhibit germinal vesicle breakdown, ultimately leading to poor embryo production (Ayoub and Hunter, 1993).

Other types of supplements, such as estrous cow serum (ECS), FBS and bovine amniotic fluid (bAF) were tested in addition to bFF to determine their effects on nuclear maturation rates (Quero et al., 1994). Preovulatory follicles were aspirated and oocytes were cultured in TCM-199 containing 20% of one of the previously mentioned protein
supplements for 24 hours. The percentage of mature oocytes was significantly higher in media supplemented with ECS (78%), FBS (79%) and bAF (77%) compared with bFF (52%). Although bFF did not yield high maturation rates, it was concluded that bovine maturation medium could be supplemented with other compounds to yield acceptable maturation rates.

Porcine follicular fluid (pFF) has also been used in maturation media to determine its effect on the ability of bovine oocytes to mature. When bovine oocytes were matured in pFF (collected from 3 to 5 mm follicles), 70% of oocytes matured compared with 75% matured in bFF (aspirated from 2 to 5 mm follicles) and 76% in TCM-199 alone. This report illustrated that certain nuclear maturation inhibitory factors found in pFF may not be exhibiting such effects on the bovine oocyte (Leibfried and First, 1980). However, when pFF was used as a supplement to mature porcine oocytes in vitro, two distinct effects were found. It was shown that pFF inhibited nuclear maturation, but it enhanced cytoplasmic maturation as indicated by pronuclear formation (Gallardo et al., 2001).

Although there are contradictory reports on the beneficial/detrimental effects of FF on nuclear maturation, Bogh et al. (2002) determined that equine follicular fluid (eFF) was beneficial when added to maturation medium. They found that (1) equine oocytes could be matured in vitro using pure equine preovulatory follicular fluid, (2) preovulatory follicular fluid collected after gonadotropin-priming was superior for supporting nuclear maturation than standard culture media and (3) equine oocytes aspirated 8 days after a previous aspiration were less likely to mature than those aspirated during the initial collection.
It is evident from previous research that *in vitro* oocyte maturation is dependent on several factors including stage of the estrous cycle, concentration of follicular fluid, the time oocytes remain in culture, various levels of inhibitory substances and the size of follicles from which the fluid is collected.
CHAPTER III

EFFECT OF HOLDING BOVINE OOCYTES IN FOLLICULAR FLUID FOR 12 HOURS AT 38ºC ON OOCYTE NUCLEAR MATURATION

Introduction

Pincus and Enzmann (1935) first reported the spontaneous nature of oocyte maturation in the rabbit following removal from the follicular environment. This original observation has since been confirmed in other mammals, leading to the general acceptance of the hypothesis that meiotic arrest in mammalian oocytes is maintained by an array of factors existing within the microenvironment of the follicle (Cho et al., 1974; Thibault et al., 1987; Bilodeau et al., 1993; De Loos et al., 1994; Aktas et al., 1995). Primary factors that have been identified include cAMP (Downs and Eppig, 1984), hypoxanthine (Downs and Eppig, 1984), E2 (Racowsky, 1985) and OMI (Tsafriri and Pomerantz, 1986). Two hypotheses have been made as to how these inhibitory substances become transmitted to the oocyte proper. First, granulosa cells are believed to secrete “arrestors” into the extracellular follicular fluid, which is either taken up directly by the oolemma and/or indirectly through the adjacent cumulus cells (Eppig and Downs, 1984). The second is that the “arrestor” is transmitted through the abundance of gap junctions known to connect both the theca and granulosa cells of the oocyte (Albertini and Anderson, 1974).

These findings become relevant when attempting to determine the effect of follicular fluid on oocyte maturation. Follicular fluid has been shown to be a beneficial supplement in the maturation media of the pig (Naito et al., 1988; Gallardo et al., 2001),
sheep (Sun et al., 1994), cow (Romero-Arredondo and Seidel, 1994; Elmileik et al., 1995; Kim et al., 1996; Choi et al., 1998; Avery et al., 2003), horse (Dell’ Aquila et al., 1997; Bogh et al., 2002; Hinrichs et al., 2002) and buffalo (Chauhan et al., 1997). However, follicular size and concentration of follicular fluid have been found to be important contributors to the success rates of oocyte maturation (Tan and Lu, 1990; Pavlok et al., 1992; Blondin and Sirard, 1994; Fouladi Nashta et al., 1998).

Utilizing follicular fluid as a holding medium for in-the-field purposes could prove to be an easy and efficient way to rescue valuable genetics from deceased females. After aspirating ovarian follicles in the field and dispensing the contents into tubes, follicular fluid could be used as a holding medium until oocytes could be recovered and IVF could be performed in a laboratory where there is essential equipment and experienced personnel. The practical considerations of this approach could prove to be beneficial in the event of the untimely death of a genetically superior animal. Schroeder et al. (1991) demonstrated that mouse oocytes were able to mature, to be fertilized and to develop into viable embryos following a 12-hour holding period following the death of the animal.

The objective of Experiment 1 was to determine the effect of bFF as a holding medium on oocyte maturation. The effect of follicular fluid was analyzed with the hopes of being able to use this medium as a potential environment in which oocytes could be held for a prolonged period of time until assisted reproductive techniques could be performed with the hopes of rescuing valuable genetic material.
Materials and Methods

Collection of Ovaries

To obtain good oocyte quality, a commercial abattoir (Hydes, Robert, LA) was contacted the morning of ovary collection. This ensured a specific window of time in which the ovaries were stored before oocyte retrieval. Ovaries were harvested from various culled female cattle of various ages, weights and reproductive stages. Immediately following slaughter, ovaries were placed into a previously prepared Ovary Transport Medium (Appendix A). Upon arrival of the technician at the abattoir, the pool of ovaries were then rinsed with fresh Ovary Transport Medium and wiped with ethanol-soaked gauze pads (4 x 4, Johnson and Johnson™, Arlington, TX) to remove blood and debris from the ovaries. The harvested ovaries were then placed into a Ziploc® plastic bag containing 500 ml of fresh Ovary Transport Medium. The ovaries were then transported ~100 km back to Louisiana State University Embryo Biotechnology Laboratory in a styrofoam container maintained at room temperature (~22ºC). The time from collection of the first set of ovaries to their arrival at the laboratory was ~6 hours.

Collection of Oocytes

Upon arrival at the laboratory, ovaries were again rinsed with fresh Ovary Transport Medium and cleansed with ethanol-soaked gauze pads. The follicle development pattern was recorded and the presence of a dominant follicle and luteal tissue were noted. Ovaries with no follicular development were excluded from the study. Follicles ranging in diameter from 2 to 9 mm were aspirated using a 20-gauge, 37.5-mm needle (Kendall, Mansfield, MA) attached to a 12-ml disposable syringe (Sherwood
The follicular aspirate from each ovary group was pooled and placed directly into sterile 15-ml plastic conical tubes (Corning, New York, NY).

**Experimental Design**

Ovaries were randomly allotted into one of two treatment groups before oocyte aspiration. Ovaries randomly assigned to Treatment A (Control Stain), which served as a control, were immediately aspirated and the recovered oocytes were denuded and stained to determine nuclear status. Ovaries allotted to Treatment B (Follicular Fluid) were aspirated and the pooled follicular fluid was dispensed into a 15-ml plastic conical tube. The tube was then placed into an incubator at 38°C with 5% CO₂ in air for 12 hours. Following the allotted time period, oocytes were denuded, stained and the nuclear status was recorded.

**Nuclear Staining Procedure**

The follicular aspirate from the two treatment groups was dispensed into 100 x 15-mm Falcon® square-style search dishes (Beckton Dickinson, Lincoln Park, NJ). Using a Nikon SMZ-2B stereoscopic microscope (Nikon, Tokyo, Japan), oocytes were recovered and then washed twice through 35 x 10-mm Falcon® plastic petri dishes (Becton and Dickinson, Lincoln Park, NJ) containing warm Oocyte Holding Medium (Appendix B) using a hand pulled 225 mm Pasteur pipette (Fisher Scientific™, Pittsburgh, PA), with an inside diameter of 100 to 150 µm. After washing the oocytes, they were placed into a 15-ml plastic conical tube containing Oocyte Denuding Medium (Appendix C). The conical tube was then placed into the incubator equilibrated at 38.5°C.
for 3 minutes to allow the cumulus cells to expand before vortexing. The tube was then vortexed for 3 minutes (Vortex Genie).

Following vortexing, 5-ml of Oocyte Holding Medium was placed into the conical tube to properly wash oocytes from the sides of the tube. After allowing 3 minutes for the oocytes to settle to the bottom of the tube, the supernatant was removed and the pellet was aspirated and placed into 500-µl of Oocyte Staining Medium (Appendix D) previously added to a four-well dish (NUNC™, Denmark). Oocytes were labeled with 1 to 5 µl/ml of nuclear stain, bis-benzimide, and held in the third well for 5 minutes while in the incubator at 38.5°C. Correspondingly, a 60 x 15-mm petri dish (Falcon®, Becton Dickinson, Lincoln Park, NJ) was prepared by adding a 60-µl droplet of Oocyte Holding Medium at the top followed by a 180-µl droplet of Oocyte Holding Medium in the center of the dish. After the allotted time period, oocytes were removed from the bis-benzimide stain and transferred into the 180-µl droplet overlaid with warm embryo-tested mineral oil (M-8410, Sigma Chemical Co., St. Louis, MO). The petri dish was then placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with Narishige micromanipulators, epifluorescent illumination and Hoffman Modulation Contrast objectives.

Holding pipettes were hand-forged from borosilicate glass tubing (1.0 mm outside diameter) using a Sutter Micropipette Puller (Sutter Instrument Co., Novato, CA) in combination with a Narishige Microforge and Narishige Microgrinder (Narishige Scientific Instrument, Tokyo, Japan). Holding pipettes were pulled to an outside diameter of 75 µm and then fire polished to create a 35-µm inner diameter. Using the
holding pipette to stabilize the oocyte, the nucleus was rotated into focus using an enucleation pipette while under exposure to UV-light. Individual oocytes were examined and their nuclear status was recorded.

Oocytes were classified as being either at the GV, GVBD, M-I or M-II stage of nuclear maturation. Oocyte quality was also recorded based on the percent of viable oocytes following treatment. A representative viable oocyte from the Control Stain (Treatment A) group is shown in Figure 3.1. Figure 3.2 illustrates non-viable oocytes following exposure to the Follicular Fluid (Treatment B) group.

**Statistical Analysis**

Data for this experiment were analyzed using the Frequency Procedure (Chi square) (SAS, 1992). The main effects and differences between treatments were considered significant at the P<0.05 level. The total number of oocytes at each stage of nuclear maturation was used as the endpoint for treatment comparison.

**Results**

The number of oocytes and percentages identified as being in the different phases of nuclear maturation are summarized in Table 3.1. There were a total of 30 ovaries aspirated per treatment group in this experiment. Of the total of 76 oocytes in the Control Stain group (Treatment A), 49% of them were identified as being at the GV stage following nuclear staining. In the Follicular Fluid group (Treatment B), 32% of the total of 112 oocytes were determined to be at the GV stage of nuclear maturation. There was no significant difference (P>0.05) between the number of oocytes identified at the GV stage in the Control Stain (Treatment A) and Follicular Fluid
Figure 3.1. A representative viable bovine oocyte from the Control Stain (Treatment A) group.
Figure 3.2. Non-viable bovine oocytes following exposure to the Follicular Fluid (Treatment B) group.
Table 3.1. Effect of holding bovine oocytes in follicular fluid on nuclear maturation rates.

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>No. of ovaries</th>
<th>No. of oocytes</th>
<th>GV (%)</th>
<th>GVBD (%)</th>
<th>M-I (%)</th>
<th>M-II (%)</th>
<th>% Viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Control Stain)</td>
<td>30</td>
<td>76</td>
<td>38 (49)(^a)</td>
<td>18 (23)(^a)</td>
<td>20 (26)(^a)</td>
<td>0 (0)(^a)</td>
<td>76 (100)(^a)</td>
</tr>
<tr>
<td>B (Follicular Fluid)</td>
<td>30</td>
<td>112</td>
<td>36 (32)(^a)</td>
<td>39 (35)(^a)</td>
<td>32 (29)(^a)</td>
<td>2 (2)(^a)</td>
<td>0 (0)(^b)</td>
</tr>
</tbody>
</table>

* A = Control Stain, B = Follicular Fluid.
\(^a,b\) Columns with different superscripts are significantly different (P<0.05) (Chi square).
(Treatment B) groups. Furthermore, the number of oocytes determined to be at the GVBD stage in Treatments A and B were not significantly different (P>0.05). The percentage of oocytes at the GVBD stage of nuclear maturation for Treatments A and B was 23% and 35%, respectively.

Similarly, the number of oocytes classified as M-I were not significantly different (P>0.05) between Treatment A (26%) and Treatment B (29%). In addition, the percentage of nuclear-mature (M-II) oocytes for Treatment A and Treatment B was 0% and 2%, respectively. There was no statistical difference (P>0.05) detected for the total number of nuclear mature-oocytes between treatment groups. However, there was a negative effect of follicular fluid (Treatment B) on the percent of viable oocytes following a 12-hour incubation period. Oocytes in Treatment B were clearly compromised, having a grainy appearing cytoplasm and often a ruptured oolemma.

**Discussion**

The number of bovine oocytes identified as being either at the GV, GVBD, M-I and M-II stages of nuclear maturation in the Follicular Fluid group (Treatment B) were markedly less than those treated with follicular fluid as reported by Avery et al. (2003). In the latter study, bovine oocytes were held in *in vitro* maturation medium consisting of either follicular fluid collected from a pool of 3 to 15 mm follicles (FF), pre-ovulatory follicles (POF), follicular fluid from heifers synchronized with a CIDR followed by PGF$_{2\alpha}$ and then oocytes were recovered using the transvaginal ultrasound-guided aspiration (TUGA) approach or TCM-199 supplemented with 5% FBS. The percent of
oocytes reaching the M-II stage was only 41% for the FF group compared with 69% from POF, 72% from TUGA and 69% from the TCM-199 group.

One possible explanation for the difference reported in maturation rates of bovine oocytes by Avery et al. (2003) could be that cumulus cells were removed from the follicular fluid by centrifugation, whereas in our study, the undiluted follicular fluid was used immediately following recovery, without any further treatment. It is possible that the cumulus cells secreted inhibitory substances, such as oocyte maturation inhibitor (OMI), hypoxanthine and cyclic adenosine monophosphate (cAMP) to ensure meiotic arrest of the oocyte before the onset of autolysis. These inhibitory substances likely remained in the follicular fluid for the 12-hour holding interval. In addition, holding these oocytes in follicular fluid for 12 hours at 38°C was detrimental to the oocytes ability to mature. Lenz et al. (1983) reported the optimal temperature for bovine oocyte maturation ranged between 35°C and 39°C. This temperature has been established for various IVM and in vitro culture media. However, incubating follicular fluid (comprised of numerous granulosa cells) within the range reported by Lenz et al. (1983) was detrimental to the oocytes and their ability to survive.

Kotsuji et al. (1994) reported that 100% of bovine oocytes matured when cultured with granulosa cells alone. However, when a combination of both granulosa and theca cells was used in the culture medium, GVBD was delayed for up to 12 hours. One can only speculate that one of the reasons that oocytes held in follicular fluid prior to maturation in our study did not mature was likely due to the combination of both follicular cell types in the fluid used to hold the oocytes.
Other factors contributing to the extremely low maturation rate found in our study in Treatment B could be the temperature at which the undiluted follicular fluid was held at prior to use, another being the affect of temperature on the cumulus cells in the follicular fluid and their ability to undergo rapid autolysis, secreting detrimental substances in the medium. Another possible factor could be the amount of time at which the oocytes remained in the fluid. A 12-hour time interval was detrimental to the oocytes as indicated by damage to the oolemma and a disrupted appearance to the cytoplasm. The percentage of viable oocytes following treatment was not comparable between Treatment A (100%) and Treatment B (0%). The detrimental effects of placing oocytes directly into pooled follicular fluid at 38°C for a 12-hour time period was dramatic (100% vs. 0%). The reason for these results remains unclear.

The ability to utilize bovine follicular fluid as a holding medium before IVM and subsequent fertilization would be beneficial for in-field purposes; however, the results from this experiment do not indicate it would be feasible to use this approach. Further research is needed to determine the reason(s) that follicular fluid exhibited a detrimental effect on bovine oocytes during a 12-hour holding period at 38°C.
CHAPTER IV

EFFECT OF HOLDING BOVINE OOCYTES IN FOLLICULAR FLUID FOR 12 HOURS AT 22°C ON SUBSEQUENT EMBRYONIC DEVELOPMENT

Introduction

Although the specific function of follicular fluid is still unclear, it has been speculated that follicular fluid could protect the oocyte from factors that could induce the premature resumption of meiosis (Espey and Lipner, 1994), assist with oocyte expulsion during the ovulatory process (Rodriguez et al., 2001), enhance sperm cell attraction to the oocyte in addition to sperm motility and the acrosome reaction (Wang et al., 2001).

One area of research has included follicular fluid as a supplement to maturation medium to determine its effect on meiotic resumption. The addition of follicular fluid to maturation medium has been used as a substitute for serum for IVP in rabbit (Kane and Headon, 1980), caprine (Guler et al., 2000), equine (Dell’Aquila et al., 1997), bovine (Leibfried et al., 1986; Kim et al., 1990; Khatir et al., 1997), porcine (Yoon et al., 2000) and human (Chi et al., 1998) species. An overall conclusion was reported from each of the above studies, although findings were generally contradictory. Consistent observations include the rates of oocyte maturation in follicular fluid were dependent on the stage of the estrous and menstrual cycles and the concentration of follicular fluid supplemented to the maturation medium.

Hinrichs et al. (2002) reported an increase in the maturation rate of equine oocytes to 24% when 100% equine follicular fluid was used in the maturation medium rather than the 12% maturation rate when 20% follicular fluid was used in their study.
Similarly, Bogh et al. (2002) found that a greater number of equine oocytes exposed to preovulatory follicular fluid during the maturation process were able to mature compared with that of control oocytes that were not exposed to follicular fluid during maturation.

Follicular fluid has also been shown to be beneficial in terms of blastocyst production. Elmileik et al. (1995) reported an increase in bovine blastocyst rates when maturation medium was supplemented with 10% follicular fluid (36%) compared with medium containing no follicular fluid supplementation (27%).

Higher maturation rates were observed for bovine oocytes cultured in follicular fluid obtained from large follicles compared with medium and small follicles (Romero-Arredondo and Seidel, 1994). Also, embryonic development to the blastocyst stage was achievable when supplementing follicular fluid in the maturation medium. However, follicular fluid was not superior to serum supplementation during the maturation process.

Even though bFF has been shown to contain various inhibitors of meiotic resumption (Eppig and Downs, 1984; Sirard and First, 1988; Gosden et al., 1990; Kadam and Koide, 1990; Sirard, 1990; Sirard and Bilodeau, 1990; Ayoub and Hunter, 1993; Downs, 1993), follicular fluid could still prove to be a beneficial medium to temporarily hold harvested oocytes for a short period of time. For example, a producer might discover that one of his highest producing dairy cows had unexpectedly died. In this situation, the producer might be able to remove the ovaries and aspirate the follicles, dispense the fluid into a tube and transport the contents to a veterinary clinic where the oocytes could be recovered for IVF. Depending on the location of the laboratory, oocytes may remain in this undiluted fluid for an extended period of time. Therefore, it would be
imperative to determine the optimal time these oocytes could be held before embryonic
developmental rates begin to decline.

The objective of Experiment 2 was to examine the effect of follicular fluid on the
\textit{in vitro} developmental potential of bovine oocytes recovered from abattoir-derived
ovaries. The specific objective was to determine the feasibility of holding bovine oocytes
in undiluted follicular fluid for a 12-hour time interval at room temperature (22°C).

\textbf{Materials and Methods}

\textbf{Ovary Collection}

To ensure that ovaries were not contaminated on the morning of collection, the
abattoir (Hydes, Robert, LA) was notified as to the time at which the ovaries would be
obtained for experimental use. The abattoir primarily slaughters dairy-type cows usually
consisting of Holstein and Jersey breeds, although various beef breeds are occasionally
processed. The weight, age and stage of reproductive cycle of the females varied
depending on the particular day of the week the ovary collections were made. However,
the day of the week was selected based on when mature, cycling cows were being
slaughtered, to assure the ovaries of immature heifers would not be used in the
experiment. Upon arrival of the technician to the abattoir, ovaries were obtained from the
plastic storage container, which had previously been filled with Ovary Transport Medium
consisting of sterile physiological saline supplemented with antibiotics (Appendix A).

The harvested ovaries were subjected to cleansing with an additional 500 ml of
fresh Ovary Transport Medium, followed by wiping them three times with ethanol-
soaked gauze pads (4 x 4, Johnson and Johnson\textsuperscript{TM}, Arlington, TX). The ovaries were
then transferred to a clean Ziploc® plastic bag containing fresh Ovary Transport Medium where they were stored at ~22°C until arrival at the laboratory. Transport time for the ovaries in this experiment ranged from 6 to 8 hours. Ovary transport time was calculated as the time from the collection of the first pair of ovaries to the time they arrived at the laboratory.

**Oocyte Collection**

Once at the laboratory, the ovaries were again rinsed with fresh Ovary Transport Medium and placed on a sterile drape. The ovaries were wiped one additional time with ethanol-soaked gauze pads to prevent contamination. The ovaries were then examined for structures that would indicate the stage of their reproductive cycle. Preovulatory follicles were identified along with the presence of any CH’s, CL’s and CA’s. Follicle populations were evaluated before randomly allocating the ovaries to their respective treatment groups. All follicles ranging from 2 to 9 mm in diameter were aspirated using a 20-gauge, 37.5-mm needle (Kendall, Manfield, MA) attached to a 12-ml disposable syringe (Sherwood Davis and Geck, St. Louis, MO). Throughout the procedure, ovaries were continuously wiped with ethanol-soaked gauze pads to reduce possible contamination. Once all usable follicles were punctured, the aspirates were pooled and placed into separate sterile 15-ml plastic conical tubes (Corning, New York, NY) that were labeled with the appropriate treatment group, the date and the aspiration time.

**Experimental Design**

Individual ovaries were distributed into either one of two treatment groups completely at random. Treatment A (Control IVF) served as the control. Ovaries
assigned to this group were aspirated and follicular fluid was pooled and placed into a 15-
ml plastic conical tube. The aspirate was searched and placed into IVM for 22 hours
followed by performing IVF. The ovaries allotted to Treatment B (Follicular Fluid) were
aspirated, followed by pooling of the follicular fluid and dispensing it into a separate 15-
ml plastic conical tube. The conical tube was properly labeled with the time, the date, the
treatment group and wrapped in aluminum foil to avoid light exposure. The conical tube
was held at 22°C for a 12-hour period. The follicular aspirate was then searched and the
oocytes were placed into a standard laboratory Maturation Medium (Appendix E)
followed by IVF.

IVM

Following follicle aspiration, the aspirate from Treatment A was immediately
dispensed into 100 x 15-mm Falcon® square style search dishes (Becton Dickinson,
Lincoln Park, NJ). The 15-ml conical tube was rinsed with 2 ml of pre-equilibrated
Maturation Medium to ensure all oocytes became dislodged from the bottom and sides of
the tube. A stereoscopic microscope (Nikon SMZ-2B, Tokyo, Japan) was used to
identify oocytes after which they were transferred into a 35 x 10-mm Falcon® plastic petri
dish (Becton and Dickinson, Lincoln Park, NJ) containing 2 ml of pre-warmed
Maturation Medium using a 225-mm Pasteur pipette (Fisher Scientific™, Pittsburgh,
PA).

Oocytes were washed twice with Maturation Medium before being placed into the
final maturation droplets. Then 10 to 15 oocytes were randomly placed into each 50-µl
droplet. Once all recovered oocytes were thoroughly washed and placed into maturation
droplets, the dish was placed into a 38°C and 5% CO₂ in air humidified incubator for 22 hours.

After the follicular aspirate had been held for 12 hours in Treatment B (Follicular Fluid), the contents of the tube were dispensed into a 100 x 15-mm Falcon® square style search dish. To loosen the pellet of cumulus cells, 2 ml of Maturation Medium were added to the tube. Recovered oocytes were placed into a 35 x 10-mm Falcon® plastic petri dish containing 2 ml of Maturation Medium, washed twice and placed into 50-µl droplets of Maturation Medium covered with embryo-tested mineral oil (M-8410, Sigma Chemical Co., St. Louis, MO). The oocytes were allowed to mature for 22 hours at 38°C and 5% CO₂ in air for 22 hours.

Media Preparation for IVF

Before oocytes were removed from the Maturation Medium, IVF stock media were prepared. Brackett-Oliphant (BO) stocks were first prepared followed by CR1aa Stock Medium as described by Rosenkrans and First (1994) (Appendices F, G and H). Then, Fertilization Medium, BO-Caffeine Medium, BSA-BO (0.6%) Medium and BSA-BO (0.3%) Medium were prepared (Appendices I, J, K and L).

Semen Preparation

After allowing for proper equilibration times, the semen was prepared for IVF. First, an empty 35 x 10-mm Falcon™ plastic petri dish was placed into the incubator labeled with the appropriate treatment group, the time and the date to avoid temperature shock to the spermatozoa. Then, a 40°C to 41°C water bath was prepared for the semen thawing process.
Semen was obtained from a fertile Holstein bull (CSS 7H5188, Genex Cooperative Inc., Shawano, WI). Semen from a single bull was used for IVF in all experiments. Once the appropriate straw of frozen semen was located, it was removed from liquid nitrogen and dropped immediately into the water bath for 45 seconds. After being thawed, the contents of the straw were dispensed into a sterile 15-ml plastic conical tube. A total of 9 ml of pre-equilibrated BO-Caffeine Medium was added to the semen. The tube was centrifuged for 6 minutes at 200 x g. The supernatant was removed, being careful to not disturb the pellet of sperm cells remaining at the bottom of the tube. An additional 9 ml of BO-Caffeine Medium was added to the tube and the pellet was resuspended. The sperm cells were then exposed to 6 more minutes of centrifugation at 200 x g. The supernatant was again removed, followed by adding 4 ml of BO-Caffeine Medium and 4 ml of BSA-BO (0.6%) Medium. The mixture was resuspended and the tube was placed in the incubator (38°C) for 10 minutes to equilibrate.

The oocytes were then removed from the Maturation Medium and placed in a 35 x 10-mm Falcon® plastic petri dish containing 2 ml of BSA-BO (0.3%) Medium. Oocytes were subjected to two washings to remove excess Maturation Medium. The dish was then placed back into the incubator at 38.5°C until the fertilization droplets were prepared. The sperm count was considered acceptable for semen use based on the total number of progressively motile sperm (≥70%).

Next, four 80-µl insemination droplets were made in the 35 x 10-mm Falcon® plastic petri dish previously labeled and placed into the incubator. Droplets were covered
with embryo-tested mineral oil and 10 to 20 oocytes were co-incubated in each insemination droplet. The dish was placed into the incubator (38ºC) for a 6-hour period.

**Removal of Oocytes from Insemination Droplets**

Before oocytes were removed from the insemination droplets, Vortexing Medium, Oocyte Wash Medium and IVC Medium (day 0 to day 3) were first prepared (Appendices M, N and O), followed by preparing 35 x 10-mm Falcon® plastic petri dishes for the fertilized ova. Oocyte Wash Medium was placed into three separate petri dishes awaiting the next step of the procedure.

Sperm-exposed ova were then recovered from insemination droplets following the 6-hour co-incubation period and were placed directly into the first dish of Oocyte Wash Medium. They were rinsed through the second dish of Oocyte Wash Medium to remove any excess insemination medium, and they were placed into the 15-ml conical tube of Vortexing Medium, followed by 3 minutes of vortexing to remove the remaining cumulus cells. Then, 2 ml of Oocyte Wash Medium was immediately added to the tube to stop the enzymatic activity of the hyaluronidase. The contents of the tube were then poured into an empty 35 x 10-mm Falcon® plastic petri dish, and the tube was rinsed with 2 ml of Oocyte Wash Medium. Sperm-exposed ova were then recovered from the dishes and placed into a final dish of Oocyte Wash Medium. The dish was placed back into the incubator (38ºC) while culture droplets were prepared.

**IVC Day 0 to Day 3**

A petri dish was labeled with the respective treatment group, the time and the date, followed by adding four, 50-µl droplets of IVC Medium covered with mineral oil.
Then, 10 to 15 embryos were transferred to each culture droplet, followed by placing the petri dish in the portable modulator incubator at room temperature (Billups-Rothenberg®, Del Mar, CA). The portable incubator was filled with a mixture of 5% CO₂, 5% O₂ and 90% N₂ at 1.4 kg/cm² (equivalent to 20 pounds per square inch; psi) for 2 minutes. The gas chamber was placed into the incubator at 38.5°C and embryonic development was recorded 72 hours post-insemination (oocytes were placed into insemination droplets on day 0).

**IVC Day 3 to Day 7**

Before evaluating embryonic development, IVC Medium (day 3 to day 7) (Appendix P) was prepared and allowed to equilibrate for 20 minutes in the incubator. Embryonic development was recorded and medium was changed at 72 hours of incubation following insemination. Embryos were assessed for cleavage patterns and evidence of degeneration. The total number of 2-cell, 4-cell, 6 to 8-cell and 16-cell embryos was recorded. In addition, embryos that appeared to have degenerated in culture were counted and then discarded. The remaining embryos were transferred to a 35 x 10-mm Falcon® plastic petri dish containing 2 ml of IVC Medium (day 3 to day 7). Embryos were washed to remove excess medium and by-products generated during the culture period.

Maturing embryos (n=10 to 15) were transferred into a 50-μl droplet of fresh IVC Medium (day 3 to day 7) covered with mineral oil. Petri dishes were then placed back into the airtight modulator incubator (room temperature) where they were again exposed to 5% CO₂, 5% O₂ and 90% N₂ for 2 minutes. The chamber was placed into a humidified
5% CO₂ incubator at 38°C until blastocyst development was recorded at 168 hours post-insemination.

Blastocyst Development

On day-7 post-insemination, embryos were removed from the incubator and the total number of embryos that reached the blastocyst stage of development were documented. Also, embryos either beginning to hatch or that had fully hatched from the zona pellucida were noted. Those embryos that developed to the blastocyst and hatching blastocyst stage were transferred to fresh IVC Medium (day 3 to day 7). Because the bovine *in vitro* developmental block occurs at the 8- to 16-cell stage (Eyestone and First, 1986), those embryos that were not able to overcome this obstacle were discarded. Petri dishes were then placed back into the triple gas mixture where embryos were left undisturbed until 216 hours post-insemination.

Hatching Blastocysts

The final time of embryo evaluation was 216 hours post-insemination. Embryos that had completely hatched from the zona pellucida were identified and recorded.

Statistical Analysis

Data for this experiment were analyzed using the Logistic Procedure (SAS, 1992). The main effects and differences between treatments were considered significant at the P<0.05 level. The total number of oocytes that developed to the 2-cell (cleaved), blastocyst and hatched blastocyst stages were used as the endpoints for treatment comparison.
Results

The total number of oocytes and percentages of embryos successfully reaching various stages of embryonic development are summarized in Table 4.1. There were a total of 120 ovaries aspirated in the Control IVF (Treatment A) and Follicular Fluid (Treatment B) groups during the experiment. The total number of oocytes inseminated from five replicates was 327 and 294 for the Control IVF (Treatment A) and Follicular Fluid (Treatment B) groups, respectively.

There was no difference in the percentage of degenerated embryos on day 3 post-insemination detected in the Control IVF group (Treatment A) (4%) compared with that of the Follicular Fluid group (Treatment B) (18%). However, a significant difference (P<0.0001) was detected between Treatments A and B for the total number of oocytes that cleaved following IVF. A total of 87% of oocytes that were inseminated underwent cleavage in Treatment A compared with only 65% of the oocytes cleaving that were assigned in Treatment B.

Similarly, of a total number of oocytes inseminated in Treatments A (327) and B (294), a significantly greater percentage (P<0.0001) of oocytes developed to the blastocyst stage of embryonic development in Treatment A (23%) compared with that for Treatment B (9%). In addition, Treatment A had a significantly greater percentage (9%) of hatched blastocysts compared with that for Treatment B (0%) following IVC for 9 days post-insemination. Images were recorded to document embryonic development in Treatments A and B (Figures 4.1 and 4.2).
Table 4.1. Effect of holding bovine oocytes in follicular fluid on embryonic development.

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>No. of ovaries</th>
<th>No. of oocytes</th>
<th>Deg (%) [72 h]</th>
<th>Cleaved (%) [72 h]</th>
<th>Blastocyst (%) [168 h]</th>
<th>Hatched (%) [216 h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>60</td>
<td>327</td>
<td>14 (4)a</td>
<td>283 (87)a</td>
<td>73 (23)a</td>
<td>28 (9)a</td>
</tr>
<tr>
<td>B</td>
<td>60</td>
<td>294</td>
<td>53 (18)a</td>
<td>192 (65)b</td>
<td>25 (9)b</td>
<td>1 (0)b</td>
</tr>
</tbody>
</table>

* A = Control IVF, B = Follicular Fluid.

 Deg = percentage of degenerate embryos.

§ Percentage of blastocysts developed from the total number of oocytes inseminated.

a,b Columns with different superscripts are significantly different (P<0.0001) (Logistic).
Figure 4.1. A representative bovine hatching blastocyst from Treatment A (Control IVF) following IVF.
Figure 4.2. Hatched bovine blastocyst from Treatment B (Follicular Fluid).
Discussion

After analyzing the results from Experiment 1, it was determined that bovine oocytes were not able to mature after being held in pooled follicular fluid at 38°C. Therefore, in this study, an additional maturation medium was used to hopefully bypass the poor maturation rates observed in Experiment 1. Results from this study indicated that oocytes could mature and become fertilized after being held in follicular fluid for 12 hours at room temperature. However, cleavage and blastocyst rates in the Follicular Fluid group (Treatment B) were compromised when compared with the Control IVF group (Treatment A). Ovary storage conditions (transport, time and temperature) from the abattoir can be discounted as a possible cause for the low developmental rates in the Follicular Fluid group (Treatment B), since good IVF embryo production resulted with the Control IVF group (Treatment A).

Choi et al. (1998) concluded that bovine oocytes could mature and subsequently be fertilized and develop into viable embryos after exposing them to follicular fluid collected from small and large follicles. Nevertheless, blastocyst production was reduced in the follicular fluid group compared with that of the control group. However, the only time oocytes were exposed to follicular fluid was through the in vitro maturation medium itself. In a similar study, Romero-Arredondo and Seidel (1996) produced viable bovine blastocysts after exposing oocytes to follicular fluid in the maturation medium. These results indicate that in vitro embryo production is possible even though oocytes have been exposed to inhibitory substances that may be present in follicular fluid for a short period of time.
Although reports have shown that follicular fluid will promote normal embryonic development, results from our study need to be considered further. For example, the time period at which oocytes remained in follicular fluid may not be optimal to promote normal embryo growth. Even though oocytes fertilized and cleaved in the Follicular Fluid group (Treatment B), further embryonic development was compromised when compared with the acceptable embryo development rate obtained in the Control IVF group (Treatment A). Of the 25 blastocysts that were produced in the Follicular Fluid group (Treatment B), only one embryo hatched out of its zona pellucida. This illustrates the unfavorable conditions to which the oocytes were exposed during the pre-maturation period. Even though the mechanism that causes this problem is not known, it is likely that a multitude of factors may be causing premature hardening of the zona pellucida. Further research is essential to determine the reason(s) causing the embryos not to hatch.

One important factor is the concentration of substances in the follicular fluid that would decrease oocyte maturation and fertilization. Hypoxanthine, oocyte maturation inhibitor and cyclic adenosine monophosphate may have negatively affected the oocytes before they were placed into a standard maturation medium, as was previously reported by Bilodeau et al. (1993). It is a possibility that these inhibitory substance(s) could also have prevented cytoplasmic maturation from occurring. The exposure of oocytes to these substance(s) for 12 hours may have been too long of a time period for subsequent hatching of embryos following IVF.

When a primary follicle becomes the dominant follicle, inhibin secretion begins suppressing FSH activity on other follicles on the same follicular wave, likely causing
their atretic demise (W.-S. et al., 1989). The underlying process of follicular atresia is apoptosis, the genetically determined death of follicle cells. Follicular atresia is a condition where a number of granulosa cells die, while others remain viable. This process undoubtedly impacts the secretion of products into the follicular fluid, ultimately impairing the developmental competence of oocytes (Jolly et al., 1994). Since oocytes in this experiment were collected from ovaries at various stages of the reproductive cycle, there is a possibility that follicular fluid from atretic follicles was aspirated and placed in the pool of follicular fluid.

Factors from these atretic follicles contribute, at least in part, to the follicular environment, which were mixed into the pool of follicular fluid to which the oocytes were exposed. Therefore, harmful substances from the process of apoptosis were being pooled with healthy follicular fluid, rendering some oocytes incapable of normal development. However, contradictory results have been reported on the effect of apoptosis on the development of IVF-derived embryos. Using an enzyme-linked immunoassay, Zeuner et al. (2003) were able to identify cumulus oocyte complexes that were recovered from healthy and atretic follicles. After adding exogenous follicular cells from atretic follicles to oocytes during IVM, there was no impact on fertilization, blastocyst formation or hatching following IVF. However, there was decreased blastocyst production when oocytes from atretic follicles were matured in a group (Blondin and Sirard, 1995).

This study was not able to illustrate the benefits of being able to hold bovine oocytes in follicular fluid for 12 hours. However, further investigations are needed to
determine the reason(s) why embryonic development was inhibited after a specific holding period in undiluted follicular fluid.
CHAPTER V

EFFECT OF HOLDING BOVINE OOCYTES IN FOLLICULAR FLUID OR LACTATED RINGER’S SOLUTION (LRS) FOR 6 HOURS AT 22ºC ON EMBRYONIC DEVELOPMENT

Introduction

Follicular fluid originates primarily from peripheral plasma, which has been modified by the metabolic activities of both the theca interna and granulosa cells (Wise, 1987). This unique fluid contains various compounds of major physiological significance, most of which are similar to compounds present in plasma. Follicular fluid contains a multitude of proteins, glycoproteins, amino acids, enzymes, carbohydrates, gonadotropins, steroids, prostaglandins, salts and immunoglobulins, all of which promote normal physiology within the follicle (Hafez, 2000).

The regulatory function of follicular fluid includes both inhibitory and stimulatory effects that exert precise control on oocyte maturation, ovulation and subsequent transport of the oocyte through the oviduct (Edwards, 1974). Inhibitory substances include OMI, luteinization inhibitor, FSH receptor-binding inhibitor and inhibin. Individual components exert their physiological effects with precise timing to ensure normal oocyte maturation, fertilization and embryonic development.

Oocyte maturation is a complex phenomenon that is still not completely understood. However, it is known that oocyte maturation involves a series of three types of physiological changes. The first includes nuclear maturation, which involves cell-cycle regulation. The second is referred to as cytoplasmic maturation, and involves both visible and nonvisible changes that take place within the oocyte during its period of
growth within the follicle. More recently, a third physiological change has been termed molecular maturation, and involves the changes that occur after nuclear condensation and are under the form of mRNA (Sirard, 2001). It is evident that follicular fluid is only a small part of the system behind the complex nature of oocyte maturation. However, functions of follicular fluid cannot be overlooked when evaluating this substance for in-the-field use or for basic research studies.

With the multiple functions attributed by follicular fluid, researchers have used this biological fluid as a component for in vitro oocyte experiments. Research has indicated that follicular fluid collected from follicles <10 mm in diameter inhibited resumption of meiosis in bovine oocytes (Ayoub and Hunter, 1993; Romero-Arredondo and Seidel, 1994). However, these findings may be beneficial for researchers wanting to utilize this fluid as merely a holding medium before oocytes could be matured using a standard laboratory maturation medium. Similarly, Aguilar et al. (2001) concluded that pure follicular fluid from small follicles (10 to 15 mm in diameter) was able to increase in vitro maturation rates of equine oocytes. In these studies, follicular fluid did not exert a detrimental effect on developmental and meiotic competence of the oocytes.

One could then hypothesize that bovine oocytes could be held in follicular fluid for a period of time without experiencing harmful effects on the oocyte. Given the results of previous research, oocytes would be prevented from maturing while in transport. This inhibitory effect on oocytes has been shown to be reversible after exposing bovine oocytes to a standard laboratory maturation medium (Mermillod et al., 2000).
Therefore, after analyzing the results from Experiments 1 and 2, an additional experiment was performed to try to successfully be able to utilize follicular fluid or Lactated Ringer’s Solution (LRS) as a holding medium. The objective of the final experiment was to determine the effect of decreasing the time and concentration of bFF on post-IVF embryonic development. The specific objective was to determine whether or not bovine oocytes could be held in follicular fluid or LRS without displaying any negative effects on subsequent embryonic developmental rates.

LRS was considered as a holding medium in addition to follicular fluid because it is a balanced (electrolyte concentration similar to serum) and isotonic (osmolality similar to serum) replacement solution that provides electrolytes in a composition similar to that of extracellular fluid. LRS is composed of NaCl, KCl, calcium and lactate, and is easily available to veterinarians and livestock producers. Since LRS is a physiologic solution, it is only logical to assume that it could be utilized as a holding medium for oocytes for a prolonged period of time.

Being able to utilize follicular fluid or LRS as a holding medium for oocytes could prove to be an advantageous protocol for producers who have experienced the untimely death of a genetically superior animal. Valuable oocytes could be saved and ultimately used to produce live offspring through the use of IVF.

Materials and Methods

Collection of Ovaries

The abattoir was notified as to the exact time of ovary collection. The abattoir confirmed the species, breed type and approximate age of the animals from which the
Ovaries were to be harvested. Ovaries were requested to be from mature, cycling cows rather than from immature heifers. Most of the ovaries used for this experiment were collected from dairy-type cows, consisting of Holstein and Jersey breeds, although a few cows of beef breeds were also processed. The abattoir immediately placed the harvested pair of ovaries in a container filled with 500 ml of Ovary Transport Medium (Appendix A).

Once the technician arrived at the abattoir, the ovaries were recovered from the holding container and fresh Ovary Transport Medium was poured into a plastic Ziploc® bag. The ovaries were then thoroughly cleansed in the medium. Then, ovaries were wiped with ethanol-soaked gauze pads to remove any remaining debris from the slaughter process. The harvested pairs of ovaries were then placed into a second plastic bag containing 500 ml of fresh Ovary Transport Medium. The ovaries were transported to the laboratory at 22°C to 25°C within 6 to 8 hours after the first set of ovaries had been collected at the abattoir.

Aspiration of Follicle Populations

Upon arrival to the laboratory, ovaries were removed from the collection bag and placed onto a sterile drape. They were once again subjected to a thorough cleansing process including wiping them with ethanol-soaked gauze pads in addition to fresh Ovary Transport Medium. Ovaries were first examined to determine the degree of follicular development and also the presence of reproductive structures, such as pre-ovulatory follicles and luteal tissue structures before assigning ovaries to treatment groups. All
harvested ovaries were utilized during the experiment except for those lacking visible follicles.

All follicles 2 to 9 mm in diameter were punctured using a 20-gauge, 37.5-mm needle (Kendall, Mansfield, MA) connected to a 12-ml disposable syringe (Sherwood Davis and Geck, St. Louis, MO). Ovaries were continuously wiped with ethanol-soaked gauze pads to prevent contamination to the ovarian surface. After all visible follicles were punctured, the aspirates were then pooled and dispensed into 6-ml, 75-mm Falcon® centrifuge tubes (Becton Dickinson, Lincoln Park, NJ). The tubes were carefully labeled with the respective treatment group, the time and the date. Aspiration of all follicles from the recovered ovaries took <45 minutes to complete.

Experimental Design

All ovaries used in the experiment were randomly assigned to either one of four treatment groups. Ovaries randomly assigned to Treatment A (Control IVF), were aspirated, the follicular aspirate was searched and recovered oocytes were placed directly into Maturation Medium (Appendix E) for 22 hours, followed by IVF. The ovaries randomly assigned to Treatment B (Follicular Fluid) were punctured and the aspirate was pooled and immediately dispensed into a 6-ml, 75-mm centrifuge tube that has been previously labeled with the respective date and the treatment group. The follicular aspirate that was recovered for Treatment C (LRS) was pooled and then placed into a different 6-ml, 75-mm centrifuge tube. Utilizing LRS in Treatment C served as a control for the Follicular Fluid (Treatment B) group. Since oocytes in Treatment C were never exposed to follicular fluid, the effect of follicular fluid on embryonic development could
be evaluated. Lastly, the aspirate recovered from those ovaries reserved for Treatment D (Follicular Fluid + LRS) was pooled and placed into a labeled 6-ml, 75-mm centrifuge tube. Then, the oocytes were placed into the 75-mm treatment tubes before the pre-maturation time interval began. Follicular aspirates from all treatment groups were held at room temperature during processing.

Experimental Procedure

The first step was to dispense precisely 3 ml of LRS (Abbott Laboratories, Chicago, IL) into a 6-ml, 75-mm Falcon® centrifuge tube. Next, the pooled follicular aspirate obtained for Treatment C was poured into a 100, 15-mm Falcon® square plastic search dish (Becton Dickinson, Lincoln Park, NJ). Oocytes were recovered and placed into a previously prepared 35 x 10-mm Falcon® plastic petri dish (Becton Dickinson, Lincoln Park, NJ) containing 2 ml of LRS using a hand pulled pipette with an outside diameter of 100 to 200 µm. Oocytes were washed through this medium before they were placed into the tube containing 3 ml of LRS.

Next, a separate 6-ml, 75-mm Falcon® centrifuge tube was prepared by mixing 1.5 ml of the LRS with 1.5 ml of follicular fluid obtained from the follicle aspiration process for Treatment D to give a final volume of 3 ml. The remainder of the aspirate was searched and recovered oocytes were placed directly into 3 ml of a combination of follicular fluid and LRS. The original tube containing the aspirate from Treatment B was left undisturbed and was not filtered while the above procedure was conducted. This allowed pellet formation of the recovered oocytes and cumulus cells. The supernatant of follicular fluid was aspirated until a final volume of 3 ml was achieved.
The three tubes from Treatments B, C and D were wrapped with aluminum foil to prevent exposure to light. They were then placed in a test tube rack where they were held for a 6-hour pre-maturation period at room temperature (22°C). After the pre-maturation period, oocytes from each group were allowed to mature in a standard laboratory Maturation Medium followed by IVF. The timeline established for individual treatment groups is presented in Figure 5.1.

Maturation of Oocytes

Follicular fluid recovered from the ovaries obtained for the Control IVF group (Treatment A) was first poured into a 100 x 15-mm Falcon® plastic dish. The tube was then thoroughly rinsed with pre-equilibrated Maturation Medium to make certain all oocytes were removed from the inside of the collection tube. Recovered oocytes were pipetted into a 35 x 10-mm Falcon® plastic petri dish containing fresh Maturation Medium using a 225-mm Pasteur pipette (Fisher Scientific™, Pittsburgh, PA). After thoroughly washing the oocytes, they were then placed into 50-µl droplets of Maturation Medium covered with embryo-tested mineral oil (M-8410, Sigma Chemical Co., St. Louis, MO). Oocytes (n=10 to 15) were put into each micro droplet. The oocytes were then placed into a humidified incubator at 38°C and 5% CO₂ in air for 22 hours.

After a 6-hour pre-maturation period for Treatments B, C and D, the tubes containing the follicular contents were individually searched and oocytes were transferred into Maturation Medium. Petri dishes were labeled with the corresponding treatment group, the time and the date. Oocytes were allowed to undergo a 22-hour maturation interval.
Figure 5.1. Timeline of the events occurring for Treatments A, B, C and D.
Preparation of Semen

Fertilization Medium, BO-Caffeine Medium, BSA-BO (0.6%) Medium and BSA-BO (0.3%) Medium were prepared prior to the start of the experiment (Appendices I, J, K and L).

After allowing the medium to equilibrate, the semen was prepared for the IVF procedure. A 35 x 10-mm Falcon® plastic petri dish was labeled and placed into the incubator to avoid temperature shock to the sperm when added. A water bath (40°C to 41°C) was prepared to thaw the straw of semen. The semen used for this experiment was obtained from two fertile Holstein bulls (CSS 7H5188 and 7H5673, Genex Cooperative, Inc., Shawano, WI). In replicates one through four, the bull identification number was 7H5188. For the remaining four replicates, semen was used from a bull with the identification number 7H5673.

Frozen sperm was instantly placed into a warm water bath for ~45 seconds to thaw. Once thawed, the contents of the straw were dispensed into a 15-ml plastic conical tube (Corning, New York, NY). Then, 9 ml of pre-equilibrated BO-Caffeine Medium was combined with the semen. The tube was then centrifuged at 200 x g for 6 minutes. The supernatant was carefully discarded, to make sure the pellet of sperm cells was not disrupted. Another 9 ml of the BO-Caffeine Medium was added to the tube, followed by gently pipetting the mixture to resuspend the pellet. The contents of the tube were centrifuged for a second time at 200 x g for 6 minutes. The supernatant was then removed and the sperm cells were washed with an additional mixture of 4 ml of BO-Caffeine Medium and 4 ml of BSA-BO (0.6%) Medium. The final combination of sperm
cells and medium were resuspended and placed into the incubator for 10 minutes for proper equilibration.

The oocytes were removed from Maturation Medium and pipetted into a 35 x 10-mm Falcon® plastic petri dish containing 2 ml of BSA-BO (0.3%) Medium. Oocytes were washed of excess medium and the dish was then placed back into the incubator until insemination droplets were prepared. To determine the number of progressively motile sperm, a count was conducted and droplets were prepared if motility was acceptable ($\geq 70\%$). Ultimately, four 80-µl insemination droplets were prepared in the previously labeled 35-mm petri dish and covered with embryo-tested mineral oil. Then, 10 to 20 oocytes were placed into each individual insemination droplet. The sperm and oocytes were allowed to co-incubate for 6 hours at 38°C and 5% CO$_2$ in air.

**Oocyte Removal from Insemination Droplets**

First, Vortexing Medium, Oocyte Wash Medium and IVC Medium (day 0 to day 3) were made for use (Appendices M, N and O). Then, 35 x 10-mm Falcon® plastic petri dishes were prepared with Oocyte Wash Medium. Sperm-exposed ova were removed from the insemination droplets and placed into the first dish containing Oocyte Wash Medium. These ova were washed twice to remove debris from the insemination process. They were then pipetted into the 15-ml plastic conical tube containing Vortexing Medium and vortexed for 3 minutes. Immediately following, 2 ml of Oocyte Wash Medium was added to the tube to halt the enzymatic activity of the hyaluronidase. The tube was then rinsed with 2 ml of Oocyte Wash Medium to obtain any embryos left behind during the procedure. Embryos were finally recovered and subjected to one more
washing. The 35-mm petri dish was placed back into the incubator until IVC droplets were prepared.

**IVC Day 0 to Day 3**

Embryos (n=10 to 15) were placed into individual droplets of IVC Medium (day 0 to day 3) covered with mineral oil. The petri dishes were placed into the portable modulator incubator (Billups-Rothenber, Del Mar, CA). A gas mixture consisting of 5% CO₂, 5% O₂ and 90% N₂ was filled into the chamber at 1.4 kg/cm², which is equivalent to 20 psi for 2 minutes. The triple gas chamber was then placed into the incubator and cleavage was assessed 72 hours post-insemination (oocytes were placed into insemination droplets on day 0).

**IVC Day 3 to Day 7**

IVC Medium (day 3 to day 7) was prepared (Appendix P) and allowed to equilibrate for 20 minutes. Embryonic development was then documented using a Nikon SMZ-2B stereoscopic microscope (Nikon, Tokyo, Japan). Embryos were assessed at the 2-cell, 4-cell, 6- to 8-cell and 16-cell stages. Embryos that had degenerated during the culture period were recorded and discarded from the experiment.

Viable appearing embryos were washed through a 35 x 10-mm Falcon® plastic petri dish containing 2 ml of IVC Medium (day 3 to day 7) using a hand-pulled pipette with an outside diameter of 200 to 250 µm to remove excess culture medium. Then, 10 to 15 embryos were pipetted into separate 50-µl droplets of IVC Medium (day 3 to day 7). Petri dishes were then placed back into the portable incubator where they were
exposed to 5% CO₂, 5% O₂ and 90% N₂ for 2 minutes. The chamber was returned to the incubator until blastocyst development could be assessed at 168 hours post-insemination.

Development of Blastocyst-Stage Embryos

Following 7 days of IVC, the total number of embryos that had reached the blastocyst stage of development were recorded. Those embryos that were hatching from the zona pellucida were also noted. In addition, blastocyst quality grades (QG) were also recorded. A QG-1 blastocyst was considered to have a tight inner cell mass (ICM) with no displaced cells and no extruded cells. A QG-2 blastocyst was considered to be an embryo that had some displaced ICM cells or extruded cells. Blastocysts that were classified as QG-3 had displaced ICM cells and extruded cells. The QG-4 classification was assigned to an embryo that displayed marked displacement of ICM cells and contained many extruded cells.

Only healthy embryos were transferred to fresh IVC Medium (day 3 to day 7) using a hand-pulled pipette (outside diameter of 250 to 300 µm) for an additional 2 days. Embryos that were not able to overcome the *in vitro* developmental block were discarded. Petri dishes were returned to the triple gas mixture until 216 hours post-insemination.

Final evaluation of *in vitro* embryo development was completed 9 days following insemination. Embryos that had completely hatched out of the zona pellucida were identified and images were recorded to denote the endpoint of the experiment.

Statistical Analysis

Data for this specific experiment were analyzed using the Logistic and Frequency Procedures (SAS, 1992). The main effects and differences were considered significant at
the P<0.05 level. The total number and percentage of oocytes that had developed to the 2-cell (cleaved), blastocyst and hatched blastocyst stages along with blastocyst quality grades were used to evaluate and compare treatments.

**Results**

The total number of oocytes and percentage of embryos reaching various stages of embryonic development following IVF are summarized in Table 5.1. Photographic images were recorded from each treatment group to show different phases of embryonic growth (Figures 5.2, 5.3, 5.4 and 5.5). In this study, 32 ovaries were aspirated to obtain oocytes for each of the four treatment groups. The total number of oocytes recovered following eight replicates was 340, 335, 297 and 310 for the Control IVF (Treatment A), Follicular Fluid (Treatment B), LRS (Treatment C) and FF + LRS (Treatment D) groups, respectively. The number and percentage of degenerated embryos for Treatments A, B, C and D were 20 (6%), 25 (7%), 101 (34%) and 60 (19%), respectively. The number and percentage of blastocysts produced during this study following 7 days of IVC were 88 (26%), 85 (25%), 14 (5%) and 48 (15%) for Treatments A, B, C and D, respectively. The number and percentage of hatched blastocysts produced following 9 days of IVC for Treatments A, B, C and D were 58 (17%), 58 (17%), 6 (2%) and 26 (8%), respectively.

There was no significant difference in embryo degeneration rates noted among the four treatment groups when observed 72 hours post insemination. There was no significant difference (P>0.05) in cleavage rates detected between the Control IVF (Treatment A) (89%) and Follicular Fluid (Treatment B) (86%) groups following IVF.
Table 5.1. The effect of bovine follicular fluid or Lactated Ringer’s Solution holding medium on subsequent bovine in vitro embryonic development.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of ovaries</th>
<th>No. of oocytes</th>
<th>Deg (%) [72 h]§</th>
<th>Cleaved (%) [72 h]</th>
<th>Blastocyst (%) [168 h]†</th>
<th>Hatched (%) [216 h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>32</td>
<td>340</td>
<td>20 (6)a</td>
<td>302 (89)a</td>
<td>88 (26)a</td>
<td>58 (17)a</td>
</tr>
<tr>
<td>B</td>
<td>32</td>
<td>335</td>
<td>25 (7)a</td>
<td>289 (86)a</td>
<td>85 (25)a</td>
<td>58 (17)a</td>
</tr>
<tr>
<td>C</td>
<td>32</td>
<td>297</td>
<td>101 (34)a</td>
<td>135 (45)b</td>
<td>14 (5)b</td>
<td>6 (2)b</td>
</tr>
<tr>
<td>D</td>
<td>32</td>
<td>310</td>
<td>60 (19)a</td>
<td>210 (68)c</td>
<td>48 (15)c</td>
<td>26 (8)c</td>
</tr>
</tbody>
</table>

* A = Control IVF, B = Follicular Fluid, C = LRS, D = FF + LRS.
§ Percentage of degenerate embryos.
† Percentage of blastocysts developed from the total number of oocytes inseminated.

Columns with different superscripts are significantly different (P<0.0001) (Logistic).
Figure 5.2. IVF-derived bovine blastocysts (day 7) from Treatment A (Control IVF).
Figure 5.3. IVF-derived bovine hatched blastocysts (day 9) from Treatment B (Follicular Fluid).
Figure 5.4. IVF-derived bovine hatched blastocyst (day 9) from Treatment C (Lactated Ringer’s Solution).
Figure 5.5. A representative IVF-derived bovine embryo (day 9) from Treatment D (Follicular Fluid + Lactated Ringer’s Solution).
However, the number of oocytes that cleaved in the LRS (Treatment C) (45%) and FF + LRS (Treatment D) (68%) groups was significantly less (P<0.0001) than the Control IVF and Follicular Fluid groups (Treatments A and B). Furthermore, there were a significantly greater (P<0.0001) number of cleaved oocytes in the FF + LRS (Treatment D) group when compared with that of the LRS (Treatment C) group.

Furthermore, there was no significant difference (P>0.05) in blastocyst formation rate 7 days post-insemination between Treatment A (26%) and Treatment B (25%). However, there were significantly fewer (P<0.0001) embryos reaching the blastocyst stage in Treatment C (5%) and Treatment D (15%) when compared with those in Treatment A (26%) and Treatment B (25%) following IVC. However, there was a greater percentage of embryos developing to the blastocyst stage in Treatment D when compared with that of the LRS (Treatment C).

In this study, there was no significant difference (P>0.05) noted in blastocyst hatching rates between the Control IVF group (Treatment A) (17%) and the Follicular Fluid group (Treatment B) (17%). However, there was a significantly lower (P<0.0001) percentage of embryos that hatched in the LRS group (Treatment C) (2%) and FF + LRS group (Treatment D) (8%) when compared with the Control IVG group (Treatment A) and Follicular Fluid group (Treatment B). Furthermore, significantly fewer (P<0.0001) embryos hatched in the LRS group (Treatment C) when compared with those in the FF + LRS (Treatment D) group. Similarly, significantly fewer (P<0.0001) embryos hatched in the LRS group (Treatment C) when compared with those in the FF + LRS group (Treatment D).
Blastocyst quality grades on 7 days post-insemination are presented in Table 5.2. There was no difference between the percentage of QG-1 blastocysts in Treatment A (43%) and Treatment B (41%) when compared with Treatment C and Treatment D. However, there were significantly fewer (P<0.05) QG-1 blastocysts in Treatment C (0%) and Treatment D (8%) when compared with Treatment A and Treatment B.

The percentage of QG-2 blastocysts was significantly less (P<0.05) in Treatment C (7%) when compared with Treatment A (44%), Treatment B (44%) and Treatment D (63%). Also, there were significantly more (P<0.05) QG-2 blastocysts in Treatment D than in Treatments A and B.

There was no significant difference (P>0.05) in the number of QG-3 blastocysts between Treatment A (13%) and Treatment B (15%). However, there was a significant difference (P<0.05) in the number of QG-3 blastocysts between Treatment B (15%) and Treatment D (29%). There were significantly fewer (P<0.05) QG-3 blastocysts in Treatment A (13%) compared with Treatment C (79%) and Treatment D (29%). Also, there were fewer QG-3 embryos in Treatment B (15%) when compared with Treatment C (79%). However, Treatment D (29%) had significantly fewer (P<0.05) QG-3 embryos than Treatment C (79%).

**Discussion**

While the IVF results produced in Experiment 2 were less than optimal for oocytes held in follicular fluid, embryo development rates in Experiment 3 was markedly increased in the Follicular Fluid group. The results from the present study indicate that
Table 5.2. The effect of bovine follicular fluid or Lactated Ringer’s Solution holding medium on blastocyst quality grades of IVF-derived bovine embryos.

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>No. of ovaries</th>
<th>No. of blastocysts</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>32</td>
<td>88</td>
<td>38 (43)(^a)</td>
<td>39 (44)(^a)</td>
<td>11 (13)(^a)</td>
<td>0 (0)(^a)</td>
</tr>
<tr>
<td>B</td>
<td>32</td>
<td>85</td>
<td>35 (41)(^a)</td>
<td>37 (44)(^a)</td>
<td>13 (15)(^a)</td>
<td>0 (0)(^a)</td>
</tr>
<tr>
<td>C</td>
<td>32</td>
<td>14</td>
<td>0 (0)(^b)</td>
<td>1 (7)(^b)</td>
<td>11 (79)(^b)</td>
<td>2 (14)(^b)</td>
</tr>
<tr>
<td>D</td>
<td>32</td>
<td>48</td>
<td>4 (8)(^c)</td>
<td>30 (63)(^c)</td>
<td>14 (29)(^c)</td>
<td>0 (0)(^c)</td>
</tr>
</tbody>
</table>

* A = Control IVF, B = Follicular Fluid, C = LRS, D = FF + LRS.
\(^{a,b,c}\) Columns with different superscripts are significantly different (P<0.0001) (Chi square).
one factor inhibiting oocyte maturation, fertilization and embryonic development was the amount of time oocytes were exposed to the pool of undiluted follicular fluid. Decreasing the holding period to only 6 hours in this experiment rather than 12 hours as in Experiment 2 proved to be favorable for oocyte viability.

The results from Experiment 2 also suggested that the high concentration of follicular fluid could have attributed to the decreased embryo development rates. However, the results recorded for the LRS group and the FF + LRS group illustrate that embryo development was significantly decreased when the concentration of follicular fluid was reduced during the holding period. This finding is similar to that of Elmileik et al. (1995), who reported that bovine blastocyst rates increased from 27% when follicular fluid was not supplemented to the maturation medium to 36% when follicular fluid was a component of the maturation medium. Similarly, Chauhan et al. (1997) also found that the an increased concentration of follicular fluid supplemented to the maturation medium of water buffalo oocytes increased the blastocyst yield from 25% when only 20% follicular fluid was used to 31% when 40% follicular fluid was supplemented to the maturation medium.

Results from our study indicate that follicular fluid was a suitable choice for a pre-maturation medium, as indicated by a greater number of excellent quality blastocysts in the Control IVF and Follicular Fluid groups compared with the LRS and FF + LRS groups. This finding suggests that the exposure of oocytes to follicular fluid during the 6-hour pre-maturation period allowed for acceptable blastocyst production and quality. Results from our study correspond with those found by Elmileik et al. (1995) who
reported that significantly more bovine blastocysts were produced following \textit{in vitro} maturation in medium supplemented with follicular fluid. When follicular fluid was not supplemented to the maturation medium, only 27\% blastocyst formation was recorded. However, when follicular fluid was supplemented to the medium, there was a 36\% blastocyst yield. These findings suggested that follicular fluid contained stimulatory substance(s) that during maturation promoted bovine oocytes to develop to the blastocyst stage following IVF.

Embryo quality was compromised in the LRS group when there was a lack of follicular fluid bathing the oocytes for the 6-hour time interval. This suggests that follicular fluid was beneficial in the holding medium, since acceptable embryo development and quality rates were obtained. This finding agrees with that of Pinto \textit{et al}. (2002), where it was concluded that bovine oocytes could be maintained in equine follicular fluid for 6 hours prior to maturation without detrimental effects on IVF-derived embryos.

Although oocytes were inhibited from maturing during the pre-maturation in follicular fluid, standard maturation protocols initiated GVBD, leading to mature oocytes that could be fertilized \textit{in vitro}. Lonergan \textit{et al}. (1997) confirmed that bovine oocytes could resume meiosis after an inhibitory period of 24 hours in cycloheximide, a known inhibitor of meiosis. The inhibitory effect was found to be fully reversible after exposing oocytes to a standard maturation medium. This finding illustrates the feasibility of \textit{in vitro} meiotic inhibition as a tool in the study of the mechanisms involved in the acquirement of developmental competence (Lonergan \textit{et al}., 1997). Furthermore, it
confirms that even though oocytes in the Follicular Fluid, LRS and FF + LRS groups may have been inhibited from maturing for 6 hours, the effects of the follicular components could be overcome by a follow-up period of IVM.

Unlike the findings in Experiment 2, this experiment illustrated the full developmental capacity of oocytes retrieved from animals that have been deceased for 6 hours. This finding coincides with a report from Blondin et al. (1997), who reported that a greater percentage of bovine blastocysts were produced from oocytes that had been held in the ovaries for at least 4 hours following slaughter. Their study supports the existing hypothesis that the developmental competence of a bovine oocyte is acquired before the oocyte ever undergoes in vitro maturation.

Although evident postmortem degenerative changes occur within the follicle and oocyte soon after the death of an animal, the culture system described in this experiment allowed for oocytes to reverse the degenerative changes that occur during maturation and subsequent fertilization. This finding is similar to a report by Schroeder et al. (1991), who found that there was a reversal of postmortem degeneration of mouse oocytes that were recovered from ovaries that had been stored for up to 6 hours following the death of the animal.

Carolan et al. (1994) reported that, on average, 15.4 embryos could be produced from oocytes harvested from a single slaughtered cow. This report clearly illustrates that a considerable number of oocytes could be rescued from terminal animals, and subsequently salvaged for use to preserve genetic material. With this in mind, the
outcome of Experiment 3 becomes beneficial from a practical aspect of rescuing oocytes from a genetically valuable female.

The results from this experiment demonstrated the feasibility of storing oocytes for a short interval to allow for transport to a veterinary clinic where oocytes could be subjected to advanced reproductive techniques. Storage of oocytes for 6 hours in follicular fluid at 22°C may be important for the rescue of oocytes from recently deceased zoological, agricultural and endangered mammals that would have otherwise been lost.
CHAPTER VI

SUMMARY AND CONCLUSIONS

Although *in vitro* embryo production systems have been improved over the last 50 years, it should be noted that the natural environment from which oocytes are obtained will likely yield better maturation, fertilization and embryonic development rates. For an *in vitro* embryo production system to become optimal, several factors must be taken into consideration. Follicle selection, oocyte quality and competence, media supplementation and culture conditions are only a few to keep in mind. Numerous experiments have been reported regarding *in vitro* fertilization systems for large and small domestic animals, however, there is still no report of any one system that surpasses the rest. The present study analyzed the effects of using undiluted follicular fluid at various times and temperatures as a holding medium for which oocytes could remain for a realistic period of time before being matured and fertilized.

The principal objective of using bovine follicular fluid as a holding medium originates from the fact that the death of a valuable animal is often an untimely event. Being able to hold oocytes in the fluid from which they initially originated could serve as a useful medium to preserve valuable genetic material until an embryo production laboratory could be available to perform advanced reproductive techniques, such as IVF.

It was evident from the results of our first experiment that this medium would be detrimental when trying to rescue oocytes from deceased females. Although nuclear maturation rates did not differ between the Control and Follicular Fluid groups, there were no viable oocytes present following their storage in follicular fluid. One
explanation for this finding would be that the temperature at which the oocytes were maintained for 12 hours was promoting various metabolic activities, which contributed to the demise of the oocytes. Another consideration is the amount of time at which oocytes were exposed to the bovine follicular fluid. This was possibly due to too many inhibitory substances in the follicular fluid over a 12-hour period of time that had a negative influence on the oocytes. Inhibitory factors may have come from autolysis of the follicular cells or simply from the metabolic by-products of the oocytes. The negative results displayed in the first experiment can only be speculated on at this time. However, further research could prove to be helpful in understanding the mechanism that causes oocytes to die in follicular fluid held at 38°C for 12 hours.

In the second experiment, follicular fluid tended to be less harmful when oocytes were held at 22°C for 12 hours. This was likely due to a decrease in the temperature at which the oocytes were exposed during the pre-maturation period. However, utilizing follicular fluid as a holding medium still proved to be suboptimal on developmental rates of IVF-derived bovine embryos. Although blastocyst formation was observed in the Follicular Fluid (Treatment B) group, the rates were significantly decreased when compared with those oocytes that had not been exposed to the *in vitro* follicular fluid environment.

An interesting observation was noted in those embryos that had developed from oocytes held in follicular fluid. Of the blastocysts that developed, only 1 of 25 embryos was able to hatch during *in vitro* culture of 9 days. The remainder were allowed to culture for a prolonged period (14 days) of time, still to find no further development.
This observation may have been due to certain substances that contributed to premature zona pellucida hardening. However, holding oocytes in follicular fluid for 12 hours at room temperature did not prove to be a worthy medium in which to hold oocytes before performing standard IVF procedures.

The final experiment of this study concluded that embryo developmental potential was not hindered when only exposing oocytes to undiluted follicular fluid for 6 hours at room temperature. With no significant differences detected between the Control IVF and Follicular Fluid groups, it was concluded that oocytes could be held in this medium without affecting an embryo’s ability to develop in vitro. Also, considering the decreased cleavage, blastocyst and hatching rates in the LRS group, it could also be concluded that follicular fluid can be beneficial to oocytes for a short period of time. Undiluted follicular fluid proved to be less detrimental compared with decreasing the concentration in half as illustrated by the decreased rates noted in the Follicular Fluid + LRS group. Although it has been shown that follicular fluid inhibits nuclear maturation, a holding period of 6 hours in follicular fluid followed by a standard in vitro maturation period can overcome the negative effects exerted by the follicular fluid.

Being able to hold oocytes in the dynamic environment of follicular fluid may prove to be useful for in-the-field purposes. The results obtained from the final study illustrated that oocytes could be maintained for up to 6 hours without damaging the ability of the oocytes to develop into a viable embryo following IVF. Producers could possibly use this technique to salvage the genetics from deceased females in order to preserve her gametes.
LITERATURE CITED


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A: OVARY TRANSPORT MEDIUM

Component (Product Information)  Amount
0.9% sterile saline  500 ml
Gentamicin (15750-060, Gibco, Grand Island, NY)  50 µl

B: OOCYTE HOLDING MEDIUM

Component (Product Information)  Amount
TL-HEPES (TLH; BioWhittaker, Walkersville, MD)  45 ml
Fetal Bovine Serum (FBS; Hyclone Laboratories, Logan, UT)  10%
Gentamicin  1 µl/ml

Place into warm water bath 20 minutes before use.

C: OOCYTE DENUDING MEDIUM

Component (Product Information)  Amount
Tissue Culture Medium-199 (Gibco Laboratories, Grand Island, NY)  10 ml
Hyaluronidase (H-3506, Sigma Chemical Co., St. Louis, MO)  0.012 g

Filter media using a 0.2-µm sterile filter (Pall Corporation, Ann Arbor, MI) attached to a 12 ml disposable syringe. Place into a warm water bath 20 minutes before use.

D: OOCYTE STAINING MEDIUM

Component (Product Information)  Amount
TL-HEPES buffer  2 ml
Bis-benzimide (Hoechst-33342; Sigma Chemical Co., St.Louis, MO)  4 µl

Wrap with aluminum foil to avoid light exposure and place into incubator 20 minutes before use.
**E: OOCYTE MATURATION MEDIUM**

<table>
<thead>
<tr>
<th>Component (Product Information)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM-199</td>
<td>8.835 ml</td>
</tr>
<tr>
<td>Bovine Follicle Stimulating Hormone (FSH; NOBL Laboratories, Sioux Center, IA) Stock</td>
<td>0.020 ml</td>
</tr>
<tr>
<td>Bovine Luteinizing Hormone (LH; NOBL Laboratories, Sioux Center, IA) Stock</td>
<td>0.125 ml</td>
</tr>
<tr>
<td>FBS</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.010 ml</td>
</tr>
<tr>
<td>Estradiol-17β (E₂; Sigma Chemical Co., St. Louis, MO)</td>
<td>0.010 ml</td>
</tr>
</tbody>
</table>

**Hormone Stocks**

FSH: 1 vial contains 490 IU (35 mg)
Reconstitute with 14 ml TCM-199 to give a 2.5-mg/ml stock (35 IU/ml)

LH: 1 vial contains 30,000 IU (8 mg)
Reconstitute with 10-ml TCM-199 to give a 0.8 mg/ml stock (3000 IU/ml)

E₂: Add 10 mg of E₂ to 10 ml of 95% ethanol to give a 1 mg/ml stock. Store at -80°C. Discard after 4 weeks.

**Medium Preparation**

1. Add 8.835 ml of TCM-199, 0.020 ml of FSH stock, 0.125 ml of LH stock, 1 ml of FBS and 0.010 ml of Gentamicin to a 15-ml conical tube (Corning, New York, NY). Sterile filter.

2. Add 0.010 ml E₂ stock.

3. Prepare two 35-mm petri dishes with 2 ml of maturation medium to be used as a wash medium.

4. Prepare 50-µl maturation droplets of filtered medium under embryo-tested mineral oil and allow to equilibrate in incubator at 38°C and 5% CO₂ for at least 3 hours.
**F: BO-A STOCK SOLUTION**

<table>
<thead>
<tr>
<th>Component (Product Information)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (S-5886, Sigma Chemical Co., St. Louis, MO)</td>
<td>4.3902 g</td>
</tr>
<tr>
<td>KCl (P-5404, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.1974 g</td>
</tr>
<tr>
<td>CaCl₂ 2H₂O (C-7902, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.2171 g</td>
</tr>
<tr>
<td>NaH₂PO₄ H₂O (S-9638, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.0743 g</td>
</tr>
<tr>
<td>MgCl₂ 6H₂O (M-2393, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.0697 g</td>
</tr>
<tr>
<td>Milli-Q Water (Millipore Corporation™, Bedford, MA)</td>
<td>500 ml</td>
</tr>
<tr>
<td>Phenol Red (0.5%) (P-0290, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.10 ml</td>
</tr>
</tbody>
</table>

Prepare the medium in a 500-ml bottle and store in the refrigerator for up to 3 months. Cover the bottle with parafilm to help maintain the pH.

**G: BO-B STOCK SOLUTION**

<table>
<thead>
<tr>
<th>Component (Product Information)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃ (S-8875, Sigma Chemical Co., St. Louis, MO)</td>
<td>2.5873 g</td>
</tr>
<tr>
<td>Milli-Q Water</td>
<td>200 ml</td>
</tr>
<tr>
<td>Phenol Red (0.5%)</td>
<td>0.04 ml</td>
</tr>
</tbody>
</table>

Prepare the medium in a 250-ml bottle. Next, inject CO₂ gas into the bottle for 1 to 2 minutes until a color change occurs. Cover the bottle with parafilm to help maintain the appropriate pH. Store in the refrigerator for no more than 3 months.

**H: CR1aa STOCK SOLUTION**

<table>
<thead>
<tr>
<th>Component (Product Information)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.6703 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.0231 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.2201 g</td>
</tr>
<tr>
<td>Pyruvic Acid (P-4562, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.0044 g</td>
</tr>
<tr>
<td>L-Lactic Acid (L-4388, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.0546 g</td>
</tr>
<tr>
<td>Glycine (G-8790, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.0039 g</td>
</tr>
<tr>
<td>L-Alanine (A-7469, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.0045 g</td>
</tr>
<tr>
<td>Milli-Q Water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Phenol Red (0.5%)</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>
Prepare the medium in a 100-ml bottle then sterile filter using a 0.2-µm filter into two 50-ml conical tubes (Corning, New York, NY). Cover the tubes with parafilm and store in the refrigerator for up to three months.

I: FERTILIZATION MEDIUM (BO-AB)

<table>
<thead>
<tr>
<th>Component (Product Information)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO-A Stock</td>
<td>38 ml</td>
</tr>
<tr>
<td>BO-B Stock</td>
<td>12 ml</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>0.00685 g</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>50 µl</td>
</tr>
<tr>
<td>Heparin Sodium (Elkins-Sinn, Cherry Hill, NJ)</td>
<td>18 µl</td>
</tr>
</tbody>
</table>

Prepare medium in a 50-ml plastic conical tube (Corning, New York, NY).

J: BO-CAFFEINE SOLUTION

<table>
<thead>
<tr>
<th>Component (Product Information)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine Sodium (C-4144, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.0243 g</td>
</tr>
<tr>
<td>BO-AB</td>
<td>25 ml</td>
</tr>
</tbody>
</table>

Prepare medium in a 50-ml conical tube, sterile filter then place into a warm water bath until needed for semen preparation.

K: BSA-BO (0.6%) SOLUTION

<table>
<thead>
<tr>
<th>Component (Product Information)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Serum Albumin (A-7511, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.03 g</td>
</tr>
<tr>
<td>BO-AB</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Prepare medium in a 15-ml conical tube, sterile filter then place into an incubator for a 15-minute equilibration period.
**L: BSA-BO (0.3%) SOLUTION**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.03 g</td>
</tr>
<tr>
<td>BO-AB</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Prepare medium in a 15-ml conical tube, sterile filter and allow for a 15-minute equilibration period in the incubator.

**M: VORTEXING MEDIUM**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM-199</td>
<td>1 ml</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>0.0010 g</td>
</tr>
</tbody>
</table>

Prepare the medium and then place into incubator prior to adding the oocytes.

**N: OOCYTE WASH MEDIUM**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM-199</td>
<td>10 ml</td>
</tr>
<tr>
<td>BSA</td>
<td>0.03 g</td>
</tr>
</tbody>
</table>

Prepare the medium, sterile filter then place into incubator for a 20-minute equilibration period.

**O: IVC MEDIUM (DAY 0 TO 3) (Rosenkrans and First, 1994)**

<table>
<thead>
<tr>
<th>Component (Product Information)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1aa Stock</td>
<td>9.5 ml</td>
</tr>
<tr>
<td>BMEaa solution (B-6766, Sigma Chemical Co., St Louis, MO)</td>
<td>200 µl</td>
</tr>
<tr>
<td>MEMaa solution (11140-050, Gibco Laboratories, Grand Island, NY)</td>
<td>100 µl</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µl</td>
</tr>
<tr>
<td>L-Glutamine (G-5763, Sigma Chemical Co., St. Louis, MO)</td>
<td>0.00146 g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.03 g</td>
</tr>
</tbody>
</table>
Prepare culture medium in a 15-ml conical tube, sterile filter and pre-equilibrate in the incubator for 20 minutes.

**P: IVC MEDIUM (DAY 3 TO 7) (Rosenkrans and First, 1994)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1aa Stock</td>
<td>9.5 ml</td>
</tr>
<tr>
<td>BMEaa solution</td>
<td>200 µl</td>
</tr>
<tr>
<td>MEMaa solution</td>
<td>100 µl</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µl</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0.00146 g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.03 g</td>
</tr>
<tr>
<td>FBS</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

Prepare culture medium in a 15-ml conical tube, sterile filter and pre-equilibrate in the incubator for 20 minutes.
VITA

Angela Marie Klumpp was born on September 22, 1979, in Metairie, Louisiana, to John and Susan Klumpp. Angela has one older brother named Michael Klumpp, who resides in New Orleans. Angela grew up in Destrehan, Louisiana, where she attended elementary school at St. Charles Borromeo Catholic School. After graduating from St. Charles Catholic High School in May 1997, she attended the University of Southwestern Louisiana where she played softball for 1 year. She then transferred to Louisiana State University in the spring of 1998 and graduated with a bachelor of science degree from the Department of Animal Sciences in December, 2001. In January of 2002, Angela entered the graduate program at Louisiana State University, under the direction of Dr. Robert A. Godke, Boyd Professor of reproductive physiology. She is a candidate for the degree of Master of Science in reproductive physiology in the Department of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.