IN VITRO MATURATION AND FREEZABILITY OF BOVINE OOCYTES

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

In recent years, it has been demonstrated by many workers that mammalian oocytes have the ability to reinitiate meiosis and complete maturation in a totally artificial environment. The oocytes are removed from the ovary and placed in a defined media under controlled environmental conditions. The media giving the best results is a balanced salt solution containing an energy source and a nitrogen source. The incubating oocytes undergo a greater degree of maturation when the oxygen concentration is reduced and the carbon dioxide concentration is increased in the atmosphere and the temperature of the in vitro system is 38 C. By using this type of controlled system, the requirements of the oocytes, such as the type of energy source utilized or the need for certain amino acids above or in place of the usual nitrogen source, can be studied by manipulating that one variable.

Preservation of embryos by freezing and storing them in liquid nitrogen has met with success in the sheep, mouse, and rabbit. The embryos were cooled slowly to about -80 C, submerged in liquid nitrogen, stored for a period of time, thawed, cultured and transferred to foster mothers. From the start of freezing to live-born young on the ground there was about a 25% success rate.

The average cow may produce about four or five calves during her life out of approximately 100,000 ova she has stored in her ovaries. As

one can see, a lot of genetic potential is being wasted. When an excellent cow leaves the herd because of old age, reproductive problems, or damage from mastitis, her ova could be collected, matured and if possible stored in liquid nitrogen until a recipient cow was available for a transplant. Freezing would eliminate the necessity of synchronizing a herd of recipients and the ova could be thawed when needed.

The purpose of this study was to characterize the maturation process of bovine oocytes and to determine their freezability.

CHAPTER II

LITERATURE REVIEW

Stages of Meiotic Chromosomes

The stages of meiosis characteristic of maturing oocytes range from the dictyate stage of prophase I to the final waiting stage of metaphase II. The primary oocytes, already formed at birth, remain dormant in the ovary of the female in the final phase of prophase I, the dictyate stage, until meiosis is re-initiated in a normal reproductive cycle. At the dictyate stage the chromatin material is either a diffuse mass or a network of chromatin fibers surrounded by a nuclear membrane. Oocytes at diplotene are also referred to as being in the germinal vesicle (G.V.) stage. Upon re-initiation of meiosis, the nuclear membrane disappears and the chromosomes begin diakinesis which is the condensation of the chromatin material into the typical short, thick, club-like shape of the metaphase chromosome (Figure 1a). It is during this stage of diakinesis that homologus pairs of chromosomes become recognizable bivalents. At the bivalent state, the chromosome number is 2n with each chromosome made up of 2 chromatids joined at a centromere. In one bivalent there are four chromatids joined together and this is referred to as a tetrad formation. When the chromosomes have finished the condensation process and have lined up at the equatorial plate, they are in the metaphase I (Figure 1b) stage ready for the subsequent anaphase I. When the metaphase I plate is split by the anaphase I movement, this division is

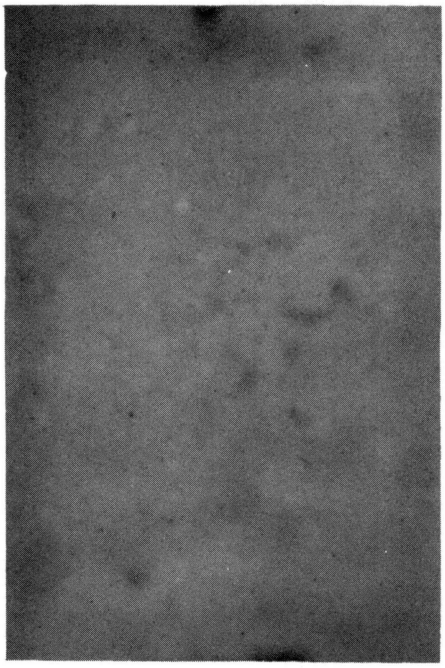

Figure 1a. G. V. Breakdown

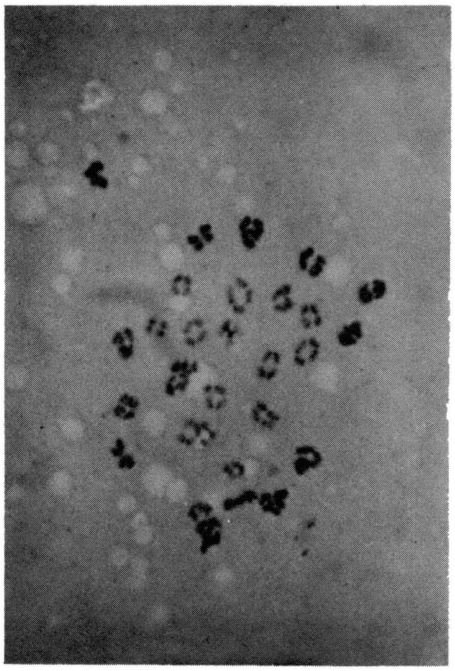

Figure 1b. Metaphase I

Figure 1c. Late Telophase I

Figure 1d. Metaphase II

known as the reduction division of meiosis. There is a division of the bivalents resulting in a reduction in the number of chromosomes, that is, each chromosome of the homologus pair goes to the opposite pole, meaning that the resulting secondary oocyte will no longer be 2n but will be in the n condition. Telophase I (Figure 1c) quickly follows the short anaphase I resulting in a secondary oocyte and the first polar body. The secondary oocyte now has only one chromosome of each homologus pair however, it still has two chromatids or the same amount of genetic material as a normal diploid cell. The next phases of meiosis consist of a short prophase II and the final metaphase II stage (Figure 1d) where the now fully matured oocyte will stay until fertilization or degeneration occurs.

Cytogenetic analyses have been done on the timing and characterization of the meiotic stages in the mouse (McGaughey and Chang, 1969) and the pig (McGaughey and Polge, 1971). McGaughey and Chang (1969) employed a newly developed air-dry technique in preparing slide preparations of the meiotic divisions of the mouse. Using PMS-HCG treated mice, they allowed the oocytes to mature in vivo and removed the eggs from the follicles at predetermined intervals before the calculated ovulation time which is 12 hours after the HCG injection. They showed that the majority of the oocytes of the treated animals had passed the prophase I stage 7 hours prior to ovulation and the metaphase I stage 3 hours prior to ovulation. The oocytes then proceded quickly through anaphase I and telophase I in the next 2 to 3 hours and finally with the large majority having progressed to metaphase II by the calculated ovulation time, 12 hours after HCG injection. McGaughey and Polge (1971) using in vitro matured pig oocytes could not give a good description of the timing of

the meiotic division because the oocytes were delayed at metaphase I due to failure of the culture media to provide nutrients necessary to support maturation. However, the description of the meiotic stages could be considered typical of the normally maturing oocytes. They showed the pig had two types of germinal vesicle stages, an earlier one composed of diffuse chromatin material and a later stage with a fibrous appearance. They distinguished prophase I from metaphase I on the basis of degree of contraction of the chromosomes and terminalization of the chiasmata. Anaphase I of the maturing pig oocytes exhibited the characteristic stretched appearance of the bivalents undergoing disjunction. Telophase I following the anaphase was distinguished from the metaphase II stage by the degree of degeneration of the polar body chromosomes, metaphase II having distinctly degenerate, undiscernible polar body chromosomes.

Culture Apparatus

A wide variety of equipment is in use to culture ova in vitro, ranging from very elaborate to very simple systems all with the final goal of regulating the external environment. The equipment typically will include an incubator for temperature control, tanks of gas to adjust the atmospheric composition and culture vessels to hold the oocytes.

Culture vessels for in vitro maturation of oocytes are almost endless, therefore the more popular types will be given. Brinster (1963) developed a method in which paraffin oil was placed in a disposable plastic tissue culture dish and small drops of culture media containing the ova were placed under the oil. The culture dish was then placed in a chamber with a controlled temperature and atmosphere. Mulnard (1965) used a slight modification of Brinster's method by putting the media

containing the ova into a capillary tube, then put this under oil. Brinster and Biggers (1965) used a petri dish containing an embryological watch glass on top of a moist sponge or glass wool to culture fallopian tubes and segments of mouse ovaries. A plastic tissue culture plate containing small numbered wells has also been used and has an advantage in that the item being cultured could easily be monitored throughout the culture period. Sterile paraffin oil was put on top of each well and a clear cover was placed over the entire plate. The standard hanging drop culture was used for culturing small numbers of cells in a small volume (Brinster, 1969). A small culture tube containing a measured volume of media and then placed open in the proper atmosphere or gassed and stoppered was used by Hammond (1949).

These culture vessels are usually overlaid with paraffin oil and placed in an airtight culture chamber within the incubator. Biggers (1965) regulated chamber and culture vessel environment with a continuous gas flow system mixing O_2 and CO_2 through flow meters. The gas was humidified by passing through two bottles of warmed water which eliminates the necessity of covering the media with oil.

External Environment

Temperature

Most of the early work to determine the optimum temperature for *in vitro* culture of mammalian cells was done on embryos. Chang (1948) stored rabbit embryos at 0, 5, and 10 C and demonstrated that development was arrested at these temperatures. Alliston (1965) showed that rabbit embryos cultivated at 40 C for six hours did not develop as well as controls incubated at 37 C when transferred into foster mothers.

Two cell mouse embryos begin to show irreversible morphological changes when temperatures reached 42 to 45 C and development was poor at temperatures of 39 to 40 C. Brinster (1969) found that at 35 to 37 C retardation of two cell mouse embryo occurred and concluded that cultures of mammalian ova should be maintained at a temperature of 37 to 38 C.

Humidity

Relative humidity of the atmosphere is important when incubating ova in media exposed to the atmosphere. It is important that the atmosphere be saturated with water vapor, otherwise, evaporation of the culture media will occur which would change the osmotic pressure of the media. Development of two cell mouse embryos, when the osmotic pressure of the culture media is raised to 308 milli-osmols (mosmol), declines about 10% compared to optimum development in a media at 276 mosmol. Haidri and Gwatkin (1973) found that 90 to 100% of hamster oocytes formed polar bodies when the osmolarity of the media was 268 mosmol. When they raised the osmolarity of the media to 278, 288 and 298 mosmol, polar body formation declined, the percentages of oocytes maturing being 80 to 90, 70 to 80 and 20 to 30 respectively. Exposure of the ova to media of reduced osmolarity resulted in no polar body formation at 258 or 263 mosmol. Research with cattle ova resulted in an 89% maturation rate in synthetic oviduct fluid media which has an osmotic pressure of 275 mosmols (Pope and Turman, 1974). As opposed to open culture conditions, the humidity of the environment does not need to be regulated when paraffin oil is layered on top of the media or when gassed and stoppered tubes are used.

Gas Composition of Culture Systems

Three major gasses, carbon dioxide, oxygen and nitrogen, are commonly used in adjusting the gas atmosphere in oocyte culture systems. Different combinations of these gasses have been employed ranging from 0% O_2, 5% CO_2 and 95% N_2 to 5% CO_2 and 95% O_2. Five percent CO_2 is beneficial for culturing mammalian ova and is routinely included as part of the gas composition (Brinster, 1969). However, there appears to be a species difference in the tolerable O_2 concentration. All mammalian species examined require O_2 for maturation. In the absence of O_2 there is no germinal vesicle (G. V.) breakdown for the first stage of maturation. Oxygen is required for oxidative processes involved with germinal vesicle breakdown as shown by the use of metabolic inhibiters in the media of rat and mouse oocytes (Zeilmaker and Verhamme, 1974; Zeilmaker et al., 1972). Oxygen concentration above that necessary for G. V. breakdown may be detrimental to the ova. Hamster ova are most sensitive to the inhibitory effects of O_2, requiring an O_2 concentration of 5% (Gwatkin and Haidri, 1974). Small increments above 5% caused a sharp decline in maturation. Mouse oocytes began to show damaging O_2 effects when the concentration was greater than 10% (Haidri et al., 1971). Other species are more tolerable of high O_2 concentrations as shown in the rat where 5% CO_2 in air did not significantly reduce the number of polar bodies extruded as compared to 5% O_2, 5% CO_2 and 90% N_2 although there was a slight trend toward better maturation at 5% O_2 (Zeilmaker and Verhamme, 1974). In the pig, concentrations of O_2 up to 95% did not block maturation (Tsafriri and Channing, 1975) although 95% O_2 did reduce the number completing maturation and increased the number of degenerating ova but did not completely inhibit maturation as seen in the

hamster and mouse.

Culture Media

Energy Source

When an energy source is included in the media, there is a significant increase in the number of ova beginning and completing maturation. However, not all energy compounds can be utilized by oocytes free of cumulus cells. Cumulus free mouse ova are capable of using pyruvate and oxaloacetate as an energy source but not phosphoenol pyruvate (PEP), glucose, malate and other sugars, while lactate is used to a limited degree. With the exception of lactate, inability to use other energy sources stems from an impermeability of the ova to them and not an enzyme deficiency of the ova (Biggers et al., 1967). Ova are permeable to lactate but it can not be used due to a deficiency of extramitochondrial nicotinamide adenine dinucleotide (NAD) (Sorenson, 1972). Pyruvate and oxaloacetate are readily utilizable by going directly into the Kreb's cycle or being changed to PEP and undergoing gluconeogenesis. The rat is somewhat different than the mouse in that some maturation occurs with bovine serum albumin as the only energy source and the rat has the ability to utilize lactate as well as pyruvate (Zeilmaker and Verhamme, 1974). Tsafriri and Channing's (1975) work with pig oocytes demonstrated that pyruvate alone promoted about 50% maturation but lactate alone failed to do so. The combination of pyruvate and lactate only increased the number completing maturation to 52%. In the absence of pyruvate and lactate, 39% of the oocytes matured to metaphase II. Possible causes for this high percentage may be due to additives that were included in the media. The pig oocytes may be able to utilize the serum

protein as postulated for the rat or the presence of glutamine in the media may have caused the maturation. In-Ha and Foote (1975) have shown that rabbit ova utilize glutamine better than pyruvate as an energy source.

pH

The pH of the media is dependent on various parameters, i.e., the energy substrate, the CO_2 concentration and the amount of bicarbonate (Brinster, 1964). Lowering or raising the bicarbonate concentration ten fold changes the pH of the media up or down, respectively, one unit. When everything else is held constant, a ten fold increase or decrease in CO_2 concentration has the same effect as bicarbonate, either raising or lowering the pH one unit. These changes in pH have a marked effect on the two cell mouse embryo's ability to develop in culture. The amount of energy substrate needed in the culture media varies with the pH. The lower the pH the lower the concentration of energy substrate necessary to obtain optimum development. Brinster (1965) found that development of two cell mouse embryos occurred in a pH range of 5.87 to 7.78 with the optimum development occurring at 7.4. This pH is utilized in most ova culturing procedures with good success.

Nitrogen Source

When a nitrogen source is added to culture media, the maturation rate of oocytes is improved. The source most commonly used in cultures is bovine serum albumin (BSA). Addition of 15% calf serum is beneficial to mouse oocyte maturation (Cross and Brinster, 1970) and the beneficial factor is in the non-dialyzable fraction of the serum. Haidri et al.,

(1971) reported that mouse oocytes can be grown in a nitrogen free media supplemented with polyvinylpyrrolidone without a significant decrease in the number of oocytes extruding the first polar body. Hamster oocytes as opposed to mouse oocytes seem to be very dependent on a nitrogen source in the media (Haidri and Gwatkin, 1973). For maximum development of hamster oocytes from preovulatory follicles, BSA plus eight additional amino acids are required *in vitro*. When hamster oocytes were cultured in BSA free media four amino acids, isoleucine, glutamine, phenylalanine and methionine were found to be essential. Bovine oocytes in a media supplemented with BSA and 10% fetal calf serum had 89% going to metaphase II as opposed to 68% when BSA and amino acids were used. The addition of amino acids to culture media containing BSA and fetal calf serum did not enhance maturation (Pope and Turman, 1974).

Follicle Cells

Studies on the contribution of cumulus cells to the maturation of oocytes have shown that there was some beneficial effect of these cells. These cells supported the maturation of the oocytes by converting unusable energy sources into pyruvate which could be metabolized by the oocytes. Mouse cumulus cells had the ability to support maturation when the only energy source in the media was either lactate, glucose or PEP (Bigger et al., 1957). Donahue (1968) showed that mouse follicle cells cultured *in vitro* produced about 0.155 mM of pyruvate from glucose which was sufficient to allow up to 88% of the mouse ova to mature.

Follicle cells also appeared to aid ova maturation in other ways not clearly elucidated. Cross and Brinster (1970) showed that when follicle cells were removed from mouse ova, there was a 20 to 30% decrease

in the number reaching metaphase II in otherwise identical conditions. Pope (Pope and Turman, 1974), using bovine ova, also demonstrated this same effect showing that 74% of the cumulus covered ova matured versus 7% of the cumulus free ova.

Hormonal Requirements *In Vitro*

Hormones have been shown to be essential for *in vivo* maturation but they appear inessential to *in vitro* development. Tsafriri et al. (1972) cultured whole rat follicles and showed that LH, prostaglandin E_2 and dibutyryl c-AMP could initiate maturation when added as opposed to no germinal vesicle breakdown when the hormones were absent. Baker and Neal (1972) induced meiosis in mouse oocytes when HCG was added to incubated follicles in an organ culture. In-Ha and Foote (1975) working with rabbit oocytes showed progesterone stimulated the oocytes to develop more rapidly in culture but neither LH nor progesterone influenced the percentage of oocytes reaching metaphase II.

When oocytes are removed from the follicle and placed into culture, it appeared as though the factors inhibiting meiosis have been removed and the oocyte then goes on and completes maturation. This is substantiated by the fact that oocytes of many different species have been matured successfully in improved defined medias without hormones. Examples of this are rabbit oocytes cultured with hormones achieved at best 85% maturation as reported by Bae and Foote (1975) versus 90% maturation in defined media (Motlik and Fulka, 1974), rat oocytes with hormones added matured 60 to 75% (Tsafriri et al., 1972) versus 85% in defined media (Niwa and Chang, 1975) and 80% of mouse oocytes matured using hormones (Baker and Neal, 1972) versus 92% in defined media (Cross and Brinster,

1970).

Success of Maturation in Farm Animals

Oocyte maturation in farm animals is quite successful in the cow (Pope and Turman, 1974), the pig (Tsafriri and Channing, 1975) and the sheep (Jagiello et al., 1974) with 89%, 80% (ova from large follicles) and 65% respectively, but the question of how viable these oocytes are remains vague. As there are no morphological characteristics for determining viability, other means of assessing the ova's state of being must be sought. One of the best ways of answering the question is to be able to fertilize the ova and have it continue development. Ova can be transferred back into foster mothers either of the same species or different species and fertilized *in vivo*. When working with the large farm animals such as the cow, foster mothers of the smaller animals are used because of the ease involved with transferring the ova and the expense. *In vitro* matured bovine ova are capable of undergoing fertilization in the oviducts of estrus gilts (17%) but not in the sheep oviduct (Shea et al., 1976). This type of work is still in its infancy and needs more research to get a good estimate of the *in vitro* matured ova's viability.

Low Temperature Storage of Embryos

In recent years, emphasis has been placed on low temperature preservation of mammalian embryos. The first mammalian embryos to be successfully frozen and thawed were those of the mouse. Whittingham, et al. (1972) using 0.3 to 2.0 C per min cooling rate with dimethylsulfoxide (DMSO) as the cryoprotective agent, froze 2500 mouse embryos and stored them for up to eight days. The embryos were slowly thawed at 4 C to

25 C per min and 50 to 70%, depending on specific rates used, of the embryos survived the freeze and progressed to the blastocyst stage upon incubation. They were transferred to pseudopregnant foster mothers of which 65% became pregnant and 40% of the embryos in the pregnant mice gave rise to normal, living, full-term fetuses or newborn mice. Whittingham and Whitten (1974) froze 85 mouse embryos at rates between 0.2 C to 1.0 C per min, shipped the embryos oversea and after a total storage time of either 189 or 240 days at -196 C, 40 to 50% of the ova originally froze developed to full-term fetuses indicating that there was no deterioration in viability when compared to the study mentioned above (Whittingham et al., 1972).

Embryos of other species have also been frozen and stored for short periods of time, then thawed and transferred to foster mothers. The methods have closely followed the procedure established by the earlier work mentioned above with a few minor changes. The results of freezing sheep embryos (Willadsen et al., 1976), although not as good as with the mouse, showed a 28% survival of sheep embryos that continued development after thawing of which 42% produced live lambs. Work with rabbit embryos (Whittingham and Adams, 1976) gave a 69 to 83% survival rate for thawed embryos developing in culture but only 7% of these transferred embryos were live born. Another study involving rabbit embryos seemed to follow a procedure similar to that for freezing sperm and it appears they were successful (Abilay and Roussel, 1976).

There has been no deep freezing work done on cow embryos but there has been some reports on storage at or around 0 C (Trounson et al., 1976a, Trounson et al., 1976b). The earlier report revealed that 8 cell embryos are irreversibly damaged upon cooling to 0 C and at the morula

stage of development only a small percentage of embryos survived cooling. The latter report stated the embryos at the blastocyst stage survived cooling much better with 92% of the embryos resuming development after storing for 30 min at 0 C. Viability of the embryos decreased to 48% after 48 hours in storage.

As can be seen from the work presented, all the freezing trials have been done with the embryo and most of the maturation studies have been done on the small lab animal. This study was initiated to (1) characterize the intermediate chromosome configurations and the time required for bovine oocytes to progress to metaphase II and (2) to determine the freezability of unfertilized bovine oocytes.

CHAPTER III

MATERIALS AND METHODS

General

This study was conducted from January 1976 to May 1977. It involved culturing bovine oocytes in a defined media to define the progressive stages of maturation and freezing the oocytes at various stages of maturation to investigate the freezability and long-term storage capabilities of the oocytes.

Collection of Oocytes

The oocytes were obtained by successive trips to either Ralph's Packing Company in Perkins or to Wilson and Company Production Plant in Oklahoma City. The ovaries were excised from the reproductive tracts and follicles approximately 1 to 5 mm in diameter were aspirated within 10 minutes. A 20 gauge x 1" needle attached to a 12 ml syringe was used to puncture the side wall of the follicle and the ova with the surrounding cumulus cells and follicular fluid were removed by aspiration. When the syringe was full, contents were expelled into a sterile 15 x 125 mm glass tube, stoppered and placed in a thermos at 38 C. The oocytes were kept in the thermos for about 3 hours, while they were being temporarily stored for transport back to the laboratory on campus. They were then removed and prepared for either culturing or freezing.

Synthetic Oviduct Fluid (SOF) Media

The media used was modified slightly from that used by Tervit, et al. (1972) which was based on the composition of the oviductal fluids of the ewe. The media modified by Pope and Turman (1974) has been shown to be highly successful for in vitro maturation of bovine oocytes. The composition of the media was as follows: 107.70 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 1.71 mM $CaCl_2$, 0.49 mM $MgSO_4$, 25.07 mM $NaHCO_3$, 3.30 mM Na lactate (Sigma, liquid ~60% solids), 0.33 mM Na pyruvate (Sigma Type II crystalline), 1.50 mM glucose, 10 g bovine serum albumin (Sigma Fraction V) per liter, 100 I. U. Na penicillin/ml, 50 µg streptomycin/ml and 10% fetal calf serum (GIBCO).

Preparation for Culture

The 15 x 125 mm tubes containing the ova and surrounding follicular contents were removed from the thermos and put into a water bath at 38 C. During the 3 hr. period, the ova had settled to the bottom of the tubes so all but 1 ml of the fluid could be aspirated out of the tubes using a disposable pipette. The remaining volume was agitated and poured into a sterile stender dish. Each tube was vigorously rinsed twice with 1 ml volumes of SOF media to remove the oocytes that had adhered to the bottom and sides of the tubes. The oocytes that had several layers of cumulus cells surrounding them were picked out of the stender dish, viewed under a stereo microscope, with a finely drawn out disposable pipette connected to a 1 ml syringe by a short piece of rubber tubing. These ova were then transferred to another sterile stender dish containing 3 ml of SOF media to rinse them from the follicular fluid and to reduce the amount of cellular debris transferred to the culture tube.

Ten to 15 oocytes were then picked out of this dish and placed into 12 x 75 mm culture tubes containing 1 ml of SOF media. The tubes were previously warmed in an oven at 38 C and equilibrated with a 5% O_2, 5% CO_2 and 90% N_2 atmosphere from a custom mixed tank of gas. After the oocytes were placed in the tubes, the tubes were re-gassed with the 5% O_2, 5% CO_2, and 90% N_2 gas mixture, stoppered and placed into the incubation oven at 38 C for maturation.

Maturation Studies

The first phase of this study utilized the procedure of Pope and Turman (1974) in further defining procedures for manipulation of bovine oocytes to determine the chromosome configuration at various times in the incubation period. A total of 432 oocytes were incubated to achieve these objectives.

Stoppered glass culture tubes gassed with 5% O_2, 5% CO_2, and 90% N_2 were incubated in an oven at 38 C for 4 to 28 hr with each tube containing 10 to 15 ova in 1 ml of SOF media. Oocytes were removed from culture starting at 4 hr after commencement of incubation, then every 2 hr thereafter except between 14 and 22 hr when they were removed every 4 hr. The oocytes were fixed on slides and the chromosomes were examined microscopically for stage of development.

Slide Preparations

After the desired period of culture, the tubes were shaken to loosen the ova from the bottom of the tube and the contents poured into an empty stender dish. Each tube was vigorously rinsed twice with a 2.0% Na citrate solution containing .1% (750 units/ml) hyaluronidase

(Sigma Type IV). The eggs in the stender dish were then picked up with the finely drawn-out pipette and placed into a 12 x 75 mm tube containing 1 ml of the citrate-hyaluronidase solution and allowed them to remain for 15 minutes. The tube was agitated periodically during this 15 min interval. After completion of this step, the contents were placed into an empty stender dish and the tube vigorously rinsed twice with citrate-hyaluronidase. The oocytes still covered with cumulus cells were placed into another tube of citrate-hyaluronidase and the above procedure repeated. When completely stripped of cumulus cells, oocytes were aspirated out of the stender dish and put into another dish containing a hypotonic solution of 0.7% Na citrate for 10 to 20 min to swell the oocytes. They were then ready to be fixed on slides using either the air dry method (McGaughey and Chang, 1969) or the in toto method (Chang, 1952).

The air dry method is the easiest and the least time consuming and therefore was used to fix 392 out of the 432 ova used in the study. Once the eggs were swollen in the hypotonic citrate solution, 5 to 20 ova were aspirated into the drawn-out pipette with fairly large, even spacings between each ova. The pipette tip was placed on a slide previously cleaned in ethanol and quickly drawn across the slide while expelling the contents. This placed the eggs in a straight line fairly well spaced on the slide. The excess hypotonic solution was removed from the slide and a 1:3 glacial acetic acid to ethanol fixing solution was dropped on top of the ova to stick them to the slide. A few extra drops of the fixative were then placed on the slide which was set aside to dry. When dry, the slide was placed in a staining dish filled with the 1:3 fix for at least 1 hour. After fixing, the slides were rinsed

in double distilled water and put into a 4% Giemsa stain for 1.5 to 2.0 hours. After staining, the slides were rinsed briefly in double distilled water and allowed to air dry. The slide was either dipped quickly into zylene, dried, then coverslipped or the coverslip was mounted on the slide with Permount without the zylene dip. Oocytes were evaluated with a bright field microscope at high power (430X) or with oil immersion (970X).

The in toto method of Chang (1952) was tedious and more difficult to use, therefore, only 40 ova were fixed this way. Instead of placing the stripped cells into the 0.7% Na citrate solution, five ova were placed in a drop between two lines of Vaseline running up and down the slide, just slightly less than the width of a 22 mm square coverslip. The coverslip was then placed over the drop and pushed down into the Vaseline until it held the ova firmly in place. A mixture of 1:3 acetic acid to alcohol was dropped on the open edge of the coverslip and allowed to flood the area under the coverslip. Thirty minutes later a 0.25% lacmoid solution in 45% acetic acid-ethanol was allowed to replace the 1:3 fix. After another 30 min, this lacmoid stain was rinsed out with 45% acetic acid-ethanol and the coverslip sealed with Vaseline. Details of the nucleus and chromosomes could be seen under bright field microscopy but phase contrast gave clearer detail.

Ova Freezing

The second major phase of this study was to determine the effects of freezing to -196 C on viability of oocytes. Extender components, cryoprotective needs and freezing protocol for successful long-term storage of sperm cells have been available for several years. However,

no such definition is available for oocytes. Oocytes in this study were frozen at several points in the incubation period. Seven hundred twenty-two oocytes were frozen in the immature (dictyate) stage, prior to any incubation, and 385, 165 and 45 oocytes were frozen at 12 hours, 14 hours, and 18 hours, respectively, after incubation to search for a possible optimum stage of meiotic division to freeze the cells.

The basic freezing procedure used was similar to that used by Whittingham et al. (1972) to freeze embryos. Ten to 40 ova were placed into a 12 x 75 mm tube with .1 ml of SOF media. The tubes were cooled to 0 C in an ice bath where 2 increments of .05 ml each of a 3M DMSO solution or 2 increments of .05 ml each of a 14% glycerol solution were added. Ten minutes were allowed for equilibration both before and after each addition. The samples were held an extra five minutes at 0 C then transferred to a seeding bath of dry ice and alcohol at -4.5 C. Each tube was seeded 2 minutes later with a minute ice crystal. The tubes were transferred to the cooling chamber for freezing to -80 C. The cooling chamber consisted of a silvered evacuated thermos liner, approximately 9 cm wide and 10 cm deep filled with 300 ml of ethanol, that sat directly in a small round styrofoam container of liquid nitrogen. This unit in turn sat inside a styrofoam chest filled with the same type of insulation. The temperature of the system was monitored by a type T thermocouple. A constant rate of cooling could be achieved by regulating the height of the liquid nitrogen around the thermos liner. A freezing rate of .33 C per minute was used to freeze 275 oocytes to an endpoint of -80 C at which point the 12 x 75 mm tubes containing the ova were put directly into liquid nitrogen and stored. Later experiments involving 325 ova used a modified procedure in which the cryoprotective agent

was added at 38 C; the cooling rate was raised from .33 C per min to .8 C per min and the ova were frozen at this new rate to an endpoint of -110 C.

A freezing procedure similar to that for bovine spermatozoa was also used. One hundred twenty-two oocytes were cooled slowly in the refrigerator in SOF media to 5 C at which time the cryoprotective agent, DMSO in a final concentration of 1.5 M or glycerol in a final concentration of 7%, was added to the freezing tubes. The ova were then transferred into 1 ml straws using a disposable pipette, sealed and placed into liquid nitrogen vapor at -180 C. The straws were then allowed to cool for 8 minutes before being plunged into the liquid nitrogen.

Ova Thawing

The rate at which frozen sperm cells are thawed has a significant influence on the viability of the cells (Senger et al., 1976; Wiggin and Almquist, 1975). This knowledge suggested that a similar situation could exist in manipulation of egg cells. Therefore, different thawing procedures were investigated within each cooling rate. The ova cooled at .33 C per min were thawed at either 4 C per min, 12 C per min. in a 0 C thaw box, or in a 38 C water bath, utilizing 120, 80, 35 and 40 ova, respectively. The ova cooled at .8 C per min were thawed at either 4 C or 12 C per min, utilizing 125 and 200 ova respectively. For ova frozen in 8 min (22 C/min), 50 ova were thawed in a 0 C thaw box and 72 ova were thawed in a 38 C water bath.

Slow warming at either 4 C or 12 C per min was achieved by placing the freezing tubes into 38 x 200 mm tubes filled with 30 or 15 ml ethanol, respectively, previously cooled to -110 C and allowing them to

warm in air.

The thawed tubes containing the ova, which had been frozen by all the above procedures, were warmed to 38 C and the cryoprotective agent was diluted out by addition of .2 ml, .2 ml and .4 ml of SOF media at 2 minute intervals. The oocytes were then placed into a stender dish, the freezing tubes were rinsed twice and 10 to 15 ova were transferred from the stender dish to culture tubes and incubated under standard conditions. The oocytes were allowed to complete the remainder of their maturation period so all treatments had a 26 hr incubation period.

CHAPTER IV

RESULTS AND DISCUSSION

Oocyte Maturation

Oocytes were incubated at 38 C in stoppered glass culture tubes containing 1 ml of SOF media equilibrated with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2 for varying periods of culture. Oocytes were removed after scheduled hr of incubation to determine the apparent time necessary for bovine cells to mature. Classification of 432 oocytes was done according to stage of meiosis on all oocytes that could be identified. There was an average of 5% of the cultured oocytes throughout the incubation periods that did not show an identifiable stage of meiotic division. The most common reason an ova could not be classified was because no chromosomes could be found in or around the ova. This could be due to either degeneration of the ova prior to or during incubation or the chromosomes were lost during the slide preparation. It was not uncommon to see a set of chromosomes adjacent to rather than within the boundaries of its respective ova. Another reason an ova was considered unidentifiable was that chromosomes were occasionally caught under the ova during fixation and they could not be clearly seen.

About 4 hrs of culture was necessary before germinal vesicle breakdown began (Table I). Once breakdown had begun, it proceeded fairly rapidly over the next 2 hr where 61% of the ova had completed this step.

TABLE I

EFFECTS OF INCUBATION TIME ON STAGE OF DIVISION OF BOVINE OOCYTES

Hr of Incubation	No. of Oocytes	Percent Oocytes in					
		Prophase I					
		Germinal Vesicle	Distinct Chrm.	Metaphase I	Telophase I	Metaphase II	Unknown %
4	39	100	--	--	--	--	0
6	31	32	61	--	--	--	7
8	36	--	96	--	--	--	4
10	21	--	62	33	--	--	5
12	19	--	53	43	--	--	5
14	19	--	--	74	10	5	11
18	20	--	--	28	44	23	5
22	21	--	--	14	38	48	0
24-26	186	--	--	3	4	87	6
28	40	--	--	5	--	88	7

By 8 hr of culture, all the eggs had lost the nuclear membrane and distinct chromosomes were present. These chromosomes were still long and fuzzy and had a banded appearance after being stained with Giemsa. Overall, the process of diakinesis or condensation of the chromatin material occurred in an 8 hr period extending from 4 hr to about 12 hr of incubation. At 14 hr, 74% of the oocytes were at the metaphase I stage. This timing of the maximum metaphase I stage agreed well with results by Jagiello et al. (1974) who also reported that an incubation period of 14 hr was necessary to achieve a good number of bovine ova in metaphase I. The main criteria for distinguishing metaphase I from the preceding stage were the degree of contraction of individual bivalents and their structure. Since the chromosomes were associated together in bivalent form due to the presence of chiasmata, a loose association would indicate terminalization of the chiasmata and therefore a later stage of meiosis. The next step in the sequence of meiosis was anaphase I which if seen would have been characterized by a stretched appearance of the chromosomes as the bivalents were being pulled apart during segregation to the opposite poles. Due to the very short duration of the anaphase I, it was not seen at any of the time periods chosen for this study. Telophase I also appears to be one of the faster steps in the divisional process. The maximum percentage of oocytes progressing to telophase I occurred in a 4 hr period between 18 and 22 hr of culture as opposed to the metaphase I stage which seemed to be more drawn out over an 8 hr period from 10 to 18 hr of incubation. This characteristic was not peculiar to _in vitro_ maturation but was also found to occur in bovine oocytes _in vivo_ as reported by Hafez and Ishibashi (1964). Telophase I could be separated into early, middle and late phases by examin-

ing the structure of individual chromosomes of the first polar body (PBI). In early telophase I, the chromosomes of PBI were similar to those of the oocyte. At middle telophase I, the PBI chromosomes were distinctly degenerate in appearance being slightly less stained and having fuzzy borders compared to the oocyte chromosomes. The PBI chromosomes of late telophase I had clumped together and become diffuse in appearance, but individual chromosomes were still visible. The final stage of maturation, metaphase II (M II) reached a maximum at 24 hr after initiation of incubation. Metaphase II was similar in appearance to late telophase I except the individual chromosomes of the PBI could not be seen. The PBI of the M II spread had a distinct characteristic appearance. No chromosomes were visible in the clump of degenerated diffuse chromatin material yet it still had a fibrous stringy nature. The time necessary for this final M II division to occur agreed with Pope and Turman (1974) who found that 89% of bovine oocytes cultured for 24 to 28 hr reached the M II stage and with Jagiello et al. (1974) who reported that 19 hr of culture was necessary before the metaphase II stage began to predominate and that by 24 hr the majority of the ova were at this stage.

Good success has been obtained with maturation of bovine oocytes in vitro. Other species have been cultured and they too have a good percentage maturing in a specific incubation period. The smaller laboratory animals usually needed a shorter duration of culture to achieve a high percentage of ova in M II. Motlik and Fulka (1974) reported that 90% of cultured rabbit ova achieved M II by 9.5 to 10.5 hr of incubation, 88 to 91% of mouse ova reached M II by 14 to 17 hr of culture (Donahue, 1968; Cross and Brinster, 1970), 13 hr of culture were needed for 85%

of rat ova to mature (Niwa and Chang, 1975) and guinea pig ova matured in 14 hr with 80 to 100% success (Jagiello, 1969). However, Yanagimachi (1975) reported only 65% going to M II in 20 to 24 hours. In contrast to the small laboratory animals, the larger farm animals needed a longer duration of culture, sheep ova being similar to the bovine and pig oocytes requiring a longer time. The M II stage of sheep ova did not begin to predominate until 23 hr of culture and overall there was 65% of the ova that arrived at M II (Jagiello et al., 1974). Pig oocytes need 43 to 48 hr for 80% of the oocytes recovered from follicles over 10 mm in diameter to reach M II. Oocytes from smaller follicles had a 25 to 65% reduction in number reaching M II (Tsafriri and Channing, 1975).

After achieving success in maturation of bovine oocytes, the normality of these cultured eggs must be considered. To evaluate this, comparison of *in vivo* to *in vitro* maturation times, cytogenetic studies and fertilization of the ova may be considered. The maturation time *in vivo* of bovine oocytes has been studied by several researchers. Hafez et al. (1963) reported an ovulation time of 24 to 26 hr following HCG injection and Christensen et al. (1974) found that the average time from the LH peak to ovulation was 28.7 hr. The attainment of M II by 24 hr as indicated in the present study would parallel the results found for the *in vivo* situation. Examination of the appearance of the chromosomes of *in vitro* matured oocytes do not differ from those reported for other species. The descriptions of the typical meiotic chromosomes of mouse (McGaughey and Chang, 1969) and pig (McGaughey and Polge, 1971) oocytes did not differ from those found in this study. Jagiello et al. (1974) noted that the chiasma frequency appeared normal in *in vitro* matured

bovine cells compared to other species. The presumtive "X" carried 3 chiasmata, the large and medium acrocentrics contained 3 chiasmata but more often only had just proximal and distal chiasmata and the small acrocentrics just had one chiasma. The number of chiasmata per type of chromosome appear to be similar between species such as the sheep (Jagiello, 1974), guinea pig (Jagiello, 1969) and human (Yuncken, 1968). Attempts to fertilize in vitro matured bovine oocytes have been done in bovine, ovine and porcine oviducts. Shea et al. (1976) matured bovine oocytes in vitro and transferred them to sheep and pig oviducts for fertilization and recovered 0 and 17% fertilized ova, respectively. Sreenan (1970) placed in vitro matured bovine oocytes into sheep oviducts which resulted in 15% of the ova becoming fertilized. Christenson et al. (1974) showed that of ova naturally ovulated and fertilized, 90.6% were fertilized. The low percentage fertilization of transferred ova in the preceding studies may not be uniquely characteristic of in vitro matured bovine oocytes. Bedirian et al. (1975) using naturally (in vivo) matured oocytes only had a 23% (5 of 22 ova) fertilization rate when these ova were transferred to bovine oviducts. This implies that the methods of the fertilization trials need to be improved to get a better estimate of the fertilizability of the ova. This becomes more apparent as rates of successful fertilization of in vitro matured ova in the smaller lab animals is examined. For example, Yanagimachi (1975) reported that 87% of in vitro matured guinea pig eggs were fertilized and Motlik and Fulka (1974) showed that 80% of rabbit ova matured in vitro could be fertilized.

The major utility value of an in vitro culture system for bovine oocytes at the present time would be found in research. Experiments

involving bovine ova are limited to small sample sizes because of the few ova that can be recovered from one cow and the time or expense involved with either operating on or sacrificing the animals. Using an in vitro maturation system, ova from cows and heifers of various ages and breeds can easily be collected in large numbers from excised ovaries at a slaughterhouse. The mixed population of animals from which the ova are obtained appears to make little difference in the percentage of ova progressing to M II. Usually, 85 to 90% of a large sample of cultured oocytes can be consistently expected to reach the final maturation stage. Also, the increase in numbers would have an advantage in that the probability of finding a statistically significant difference due to treatments would be greatly improved. One such use of the system would be in vitro fertilization trials where the ability of particular agents to capacitate sperm could be investigated. The low variability in terms of numbers of ova progressing to M II and the advantage of numbers would make the in vitro system desirable.

Ova Freezing

It is known that cooling cells may be deleterious to further development, therefore, before the freezing trials were conducted, preliminary tests were done to determine if cooling the immature ova to 0 C and addition of the cryoprotectant agent at 0 C had an adverse effect on the viability of the ova. Immature oocytes stored at 0 C for 6, 18, 24, and 48 hr with 10 to 15 oocytes per storage time seemed to be affected little by this temperature (Table II). There was a slight increase in unreadable cells probably due to degeneration, however, even after 48 hr at 0 C, 70% of the oocytes matured to M II after 24 hr of

culture. This is in contrast to a study by Wilmut et al. (1975) who showed that storage of early bovine embryos of 8 cells or less at 0 C was deleterious. Another small study was done to determine the effects of DMSO on the ova at 0 C. The ova were held at 0 C for 30 minutes while being equilibrated with DMSO, then this was followed by a short 5 min period at -4.5 C where the surrounding media containing the oocytes was frozen by seeding with a small ice crystal. Of the 10 eggs used, all 10 went on to mature to the M II stage. Therefore, it appears that the cryoprotective agents, cooling to -4.5 C, or extended holding at 0 C has little effect on viability of oocytes.

TABLE II

EFFECTS OF 0 C STORAGE ON IMMATURE BOVINE OOCYTES*

Hr. of Storage	Number of Oocytes in			
	Metaphase I	Telophase I	Metaphase II	Unknown
6	1	1	8	3
18	-	2	7	4
24	-	4	7	4
48	-	-	8	3

*All treatments incubated for 24 hours after storage.

Several different freezing protocols were utilized in investigating the storage of oocytes at -196 C in a viable condition. The first series of trials utilized 275 immature ova by adding either DMSO or glycerol

at 0 C, using a .33 C per min. freeze, then thawing batches of ova at either 4 C per min., 12 C per min., thawing in a 0 C thawbox or in a 38 C waterbath. After thawing to 38 C, the cryoprotective agent was diluted with SOF media. As seen from Table III, there was no cell that continued with maturation when placed in culture under standard conditions. The thawed cells that were placed into culture had a very loose cumulus mass around them and these cumulus cells were easily knocked off the ova as opposed to the tight mass of firmly attached cumulus cells normally seen around an immature ova. These loose cumulus cells were not sticky as is characteristic of loose cells seen at the end of a 24 hr culture period. Not one ova had cumulus cells around it at the end of culture regardless of the fact that the majority of the cells had a cumulus covering at the start of incubation. The properties of the zona pellucida of the thawed ova were changed so that the zona was more easily fractured upon agitation. After the ova were fixed on slides, some of the ova stained a blotchy reddish pink-blue color instead of the characteristic homogenous pink color and no chromosomes could be found in any of these oocytes. Due to the failure to mature any of the oocytes, a second series of trials were initiated, the only modifications being to add the cryoprotective agent at 38 C, to increase the cooling rate from .33 C per min. to .8 C per min. and to thaw at either 4 C per min. and 12 C per min. This also resulted in failure as all 325 ova used in the trial became degenerate.

Another trial using immature ova was conducted using the procedure of Abilay and Roussel (1976) for freezing embryos. A standard semen freezing technique was used on the ova to test the effects of a more rapid freeze. The ova were frozen to approximately -180 C in 8 minutes

TABLE III

SUMMARY OF EFFECTS OF FREEZING ON BOVINE OOCYTES

Treatment	DMSO		Glycerol	
	No. Oocytes	No. Maturing	No. Oocytes	No. Maturing
1. Freeze at .3 C/min.	130	-	145	-
Thaw at 4 C/min.	55	0	65	0
12 C/min.	40	0	40	0
in 0 C thawbox	15	0	20	0
in 35 C waterbath	20	0	20	0
2. Freeze at .8 C/min.	125	-	200	-
Thaw at 4 C/min.	50	0	75	0
12 C/min.	75	0	125	0
3. Freeze 8 min. to -180 C	50	-	72	-
Thaw in 0 thawbox	20	0	30	0
38 C waterbath	30	0	42	0
4. 12 hr. preincubation - .8 C/min. freeze	105	0	180	0
14 hr. preincubation - .8 C/min. freeze	60	0	105	0
18 hr. preincubation - .8 C/min. freeze	0	0	45	0

in liquid nitrogen vapor and thawed either in a 0 C thawbox or in a 38 C waterbath. The condition of these cells post-thaw was the same as those mentioned above in the slower freeze trials except all the ova stained the blotchy blue-red. All 122 ova degenerated in culture.

Since none of the freezing protocols were successful with immature ova, several trials with partially matured oocytes were conducted. Oocytes were incubated for either 12, 14 or 18 hr prior to freezing them at .8 C per minute and thawing at 12 C per minute. These hours were chosen based on the previous maturation studies to allow the greatest chance to observe a small amount of development if it occurred. However, all of the 285, 165 and 45 oocytes incubated for 12, 14 and 18 hrs, respectively, were non-viable after freezing (Table III). Again the condition and appearance for these ova was no different than mentioned above. For the ova that stained a homogenous pink, maturation of the oocytes appeared to be halted as evidenced by no further progression of the post-thawed oocyte chromosomes toward M II upon reincubation and the large amount of chromosomal degeneration present per ova.

One reason for the failure of the ova to survive the freezing process and mature in culture may be due to the need to freeze two different size cells at the same time, i.e., the cumulus cells and the oocyte. Bovine oocytes are 150 to 200 μm while cumulus cells are 15 to 20 μm in diameter. Pope and Turman (1974) have demonstrated that oocytes need to be surrounded by the support cells or cumulus cells in order to mature *in vitro*. It is not known what specific role the cumulus cells play in maturation but it is known that they are able to convert glucose and lactate to pyruvate so possibly they supply some other necessary substrate(s) that the ova requires to mature. Their study (Pope and

Turman, 1974) showed that oocytes without cumulus cells only had 7% reach M II and 86% remained at the dictyate stage compared to controls with cumulus cells in which 74% of the ova progressed to M II and only 7% were left at dictyate. From this it is speculated that the freezing process interrupts the connections between the ova and the cumulus cell by either damaging the ova or the follicle cells. It is known that there is an optimum freezing and thawing temperature for different size cells (Mazur and Schmidt, 1968). The rate of freeze depends on the volume of the cell and how permeable the cell membrane is to water. The larger the cell and/or the less permeable the cell membrane is to water, the slower the freeze must be for optimum recovery. The optimum freeze rate is slow enough to allow the cell to dehydrate sufficiently to remain in vapor pressure equilibrium with the frozen external ice to avoid intracellular freezing but fast enough to avoid toxic solution effects. The optimum freezing rate for the embryo at about 0.8 C per min (Whittingham et al., 1972) is probably too slow for the smaller cumulus cells because of damage due to solution effects. There is also an optimum thawing rate for each cell size to prevent damage to the membrane by recrystallization (Miller and Mazur, 1976). The slow thaw of about 12 C per min. shown to be optimum for embryos (Whittingham et al., 1972) is too slow for the cumulus cell which is damaged more. Because of the dependence of the ova on the follicle cells and the fact that the follicle cells probably do not survive the freezing and thawing process, maturation of the ova is prevented. It would be speculation at this point as to the temperature at which the ova is killed. It may well be that the range from 0 C to -15 C would be feasible temperatures to store the immature ova at least for 2 days and possibly longer. It

is apparent that the freezing characteristics and requirements of oocytes differ very greatly from spermatozoa.

It was assumed that a freezing rate of 0.8 C per min and a thawing rate of 12 C per min was correct procedure and it was hoped that a partially matured ova before freezing would not be so dependent on the cumulus cells during the subsequent incubation. From the results, this did not seem to be the case. Either the assumption was incorrect and a slower cooling rate was needed to successfully freeze the large ova or the partially matured ova had changed some of its properties and was more sensitive to low temperatures as shown for the early bovine embryos. The freezing of fully matured oocytes is hampered by the fact that there is no good way to test viability post-thaw. A successful method of fertilizing these matured oocytes would first have to be worked out so viability of the oocytes could be tested in large numbers.

More work is needed in the area of ova freezing to determine the requirements necessary for low temperature preservation of the bovine oocytes. From the results of this study, it does not appear as though the standard embryo freezing techniques are applicable to immature or partially matured ova. The factors that must be solved are the temperature that kills the oocytes, the optimum freezing and end-point temperatures, thawing temperature and the kind and amount of cryoprotective agent to obtain the most successful results.

CHAPTER V

SUMMARY

The objectives of this study were: (1) to characterize the intermediate chromosome configurations and the time required for bovine oocytes to progress to M II; (2) to determine the freezability of unfertilized bovine oocytes.

A series of maturation trials were performed on 432 immature bovine oocytes. The oocytes were obtained from local slaughter houses by aspirating the follicles of the excised ovary using a 20 gauge needle attached to a 12 ml syringe. The oocytes were kept at 38 C in a thermos and trnasported back to the laboratory where they were placed immediately into culture. The oocytes were cultured for 4 to 28 hrs in modified SOF media containing 10 gm bovine serum albumin per liter and 10% fetal calf serum. The oocytes were incubated in 12 x 75 mm stoppered glass tubes equilibrated with 5% O_2, 5% CO_2 and 90% N_2 at 38 C. Upon completion of the desired incubation time, the oocytes were fixed on microscope slides, stained with Giemsa stain and examined to determine the stage of meiosis they had reached.

A high percentage of bovine ova may be expected to go from prophase I to M II in vitro. After 4 hr of culture, the germinal vesicle (G. V.) begins to breakdown and by 8 hr of culture all G. V. have disappeared. The process of diakinesis begins after breakdown and is the prevalent stage for about 8 hr of culture. The chromosomes contract during this

time and become visible. The prophase I chromosome is still long and fuzzy and appeared to be banded when stained with 4% Giemsa. The chromosomes were considered to be in metaphase I when the degree of contraction was the greatest and when terminalization of the chiasmata occurred. Metaphase I occurred at about 12 hr of culture with 74% of the oocytes being at this stage after 14 hr of incubation. The remaining stages of the meiotic division proceded fairly rapidly. Telophase I appeared to dominate at about 18 to 22 hr of culture and ova in M II reached a maximum of 88% at 24 hr of culture. The main criteria for distinguishing between telophase I and M II was based on the appearance of the first polar body. If there were no discernable chromosomes present in the chromatin mass of the polar body, then the ova was considered to be in the M II stage. This timing sequence agrees well with in vivo findings.

The freezability of oocytes was investigated utilizing four procedures and 1217 ova. The first procedure utilized a .33 C per min. freezing rate on 275 immature ova protected with either DMSO or glycerol and thawed them at either 4 C per min., 12 C per min or in a 0 C or 38 C waterbath. The second procedure was similar to the first except that the 325 ova used were frozen at a rate of 0.8 C per min and thawed at either 4 C or 12 C per min. The third procedure used was a standard semen freezing technique for straws. The 122 immautre ova protected with either glycerol or DMSO were placed into liquid nitrogen vapor at -180 C or 8 min then stored in liquid nitrogen. The straws were thawed in a 0 C or 38 C waterbath. The fourth procedure utilized the .8 C per min freezing rate and the 12 C per min thawing rate on a total of 495 partially matured oocytes that were preincubated for either 12, 14, or 18 hours.

After the ova of all procedures were thawed and the cryoprotective agent was diluted out at 38 C by a series of dilutions, the oocytes were placed into standard culture conditions which consisted of SOF media in stoppered glass tubes gassed with 5% O_2, 5% CO_2, and 90% N_2 and incubated at 38 C. All treatments received a total incubation time of 26 hours.

None of the ova frozen in the immature stage showed any evidence of resumption of development when placed into culture after thawing. Several reasons could have been responsible for the lack of maturation. It is known that bovine oocytes require the follicle cells to supply necessary factors to support maturation to M II in vitro. The freezing process seemed to have damaged either these cells or their inter-connections with the oocytes as evidenced by the fact that they were extremely easy to knock off the ova after thawing whereas they should have been firmly attached in a compact mass around the immature ova. Failure of the follicle cells to remain attached may be due to the freezing and thawing rates used. It has been shown that different size cells require different freezing and thawing rates. The slow freezing rates of .33 C and .8 C per min. probably damaged the follicle cells while the fast 8 min freeze and thaw in a 0 C or 38 C waterbath damaged the ova. To try to overcome these problems associated with the immature ova, partially matured ova 12, 14, or 18 hr were frozen at a rate of 0.8 C per min., thawed at 12 C per min and reincubated for the remainder of the 26 hr period. These ova also failed to resume maturation when culturing was completed.

The appearance of the fixed ova after the last incubation was fairly typical throughout all treatments. Of the immature ova frozen at

.33 C or .8 C per min and thawed at 4 C or 12 C per min., the majority were a typical homogenous pink color lacking any signs of chromatin material. A few cells were a blotchy blue-pink color also lacking any visible chromatin material. The immature ova frozen in 8 min and thawed in a 0 C or 38 C waterbath were all blotchy blue-pink in appearance. The partially matured cells usually stained the normal pink color but the chromatin material was either absent or highly degenerate.

The results of this study indicate that bovine ova matured in vitro develop normally, however, freezing and low temperature storage (-196 C) of bovine ova is not feasible at this time.

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