BOVINE IN VITRO EMBRYO PRODUCTION AND OOCYTE PRESERVATION

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Animal Science)

We accept this thesis as confirming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December 1999

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The University of British Columbia
Vancouver, Canada

Date 16/02/2000

DE-6 (2/88)

ABSTRACT

Several experiments were done with the overall objective to improve the production of in vitro embryos. In the first experiment, the optimum period for ovary removal and the effect of FSH priming at standing estrus were investigated to maximize viable oocyte yield from culled cows. Animals were ovariectomized either 2 d after induced estrus, 2 d after a single dose of FSH on the day of standing estrus or randomly irrespective of the stage of the estrous cycle. Oocyte recovery and, cleavage and blastocyst formation rates were recorded for each treatment. Highest oocyte recovery was obtained from FSH-primed cows. Cleavage and blastocyst formation rates were not significantly different among treatments. These results indicated that FSH treatment increases oocyte yield in culled cows. The second experiment examined methods for the short-term preservation of bovine oocytes. Oocytes were matured in either i) straws containing maturation media (MM) at 38.5 °C without 5% CO_2, ii) straws containing MM at room temperature without 5% CO_2 or iii) culture plates containing MM at 38.5 °C and 5% CO_2. After 24 h, oocytes were fertilized and cultured in vitro. Oocytes, which were matured at room temperature without 5% CO_2, showed significantly lower maturation, fertilization, cleavage, and blastocyst formation rates than those matured at 38.5 °C. Oocytes matured at 38.5 °C with or without 5% CO_2 showed similar maturation, fertilization and cleavage rates, however, those with 5% CO_2 showed a significantly higher blastocyst formation rate. Therefore, 38.5 °C and a 5% CO_2 environment are optimal for oocyte maturation and embryo development. The third experiment was done to find a suitable cryopreservation method for the long-term

preservation of bovine oocytes. Developmental competency of immature and mature oocytes undergoing slow freezing or vitrification was measured by cleavage and blastocyst formation rates. None of the slow frozen mature oocytes or vitrified immature and mature oocytes cleaved 72 h after insemination or developed to the blastocyst stage. Although some slow frozen immature oocytes eventually cleaved, the cleavage rate was significantly lower than that of control oocytes. This indicated that both cryoprotectant and cooling procedure affect the developmental competency of bovine oocytes.

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ABBREVIATIONS

AFGP	– Antifreeze glycoprotein
ANOVA	– Analysis of variance
BO	– Brackett and Oliphant medium
BSA	– Bovine serum albumin
COC	– Cumulus oocyte complex
DMA	– Dimethylacetamide
DMSO	– Dimethylsulfoxide
DPBS	– Dulbecco's phosphate buffered saline
EDTA	– Ethylenediaminetetraacetic acid
EG	– Ethylene glycol
EGF	– Epidermal growth factor
EGTA	– Ethylene glycol tetra-acetic acid
ET	– Embryo transfer
FGF	– Fibroblast growth factor
FSH	– Follicle stimulating hormone
G	– gauge
gm	– Gram
g	– Gravity
GnRH	– Gonadotropin releasing hormone
GV	– Germinal vesicle
h	– Hour
HEPES	– *N*-(2hydroxethyl)-piperazine-*N'*-(ethanesulphonic acid)
IGF-I	– Insulin like growth factor - I
IGF-II	– Insulin like growth factor - II
IVF	– In vitro fertilization
LH	– Luteinizing hormone
M	– Molar
mg	– Milligram
min	– Minutes
ml	– Milliliter
MM	– Maturation media
mm	– Millimeter
mM	– Millimolar
µg	– Microgram
P_4	– Progesterone
PDGF	– Platelet derived growth factor
$PGF_{2\alpha}$	– Prostaglandin $F_{2\alpha}$
PROH	– 1,2 Propanediol
PVP	– Polyvinylpyrolidone
RNA	– Ribonucleic acid
SCS	– Superovulated cow serum
T	– Trehalose
TALP	– Tyrode's albumin lactate pyruvate
TCM199	– Tissue culture medium 199

ACKNOWLEDGEMENTS

I would like to express my appreciation and gratitude to Dr. R. Rajamahendran for consenting to be my advisor and for his encouragement, guidance and support at every stage of my thesis research. His valuable advises and suggestions stimulated my interest in the field of in vitro embryo production.

I am much thankful to my advisory committee members Dr. J.A. Shelford, Dr. K.M. Cheng and Dr. D. Kitts, and Dr. J.R. Thompson for their supportive suggestions for the completion of my thesis.

I gratefully appreciate all the technical assistance rendered at various stages of my graduate training by Gills Galzy, Sylvia Leung, K. Sivakumaran and Luz Aquino. I am much thankful to Shenaz Motani and Donna Bailey who took care of my office requirements. I appreciate Kunanithy for collecting ovaries for in vitro embryo production studies from slaughterhouse at Langley.

I gratefully acknowledge my fellow graduate students Lisa Stephens, Mohan Mahesh, Ming Yang, Pavaneesh Madan, Mohamod Tagi, Afsaneh Motamed, Hirad Mohamed, Nagarajan, Divakar Ambrose and Jamal Kurutu for their suggestions and other helps in writing and correcting my thesis and scientific papers. My very special thanks must be addressed to my friend Rajeswaran for his assistance in the statistical analysis of data. I am also much thankful to my friends Marcus Samuel, Premsiri, Thavaraja, Sivathayalan and Farhan for giving me moral support during my program.

I would like to thank all the faculty and staff members of the Department of Animal Science. I am particularly grateful to Dr. D.M. Shackleton for the assistance in the preparation of my presentations. I am much grateful to Faculty of Agricultural Sciences for offering me teaching and research assistantships and Faculty of Graduate Studies for awarding a travel grant.

I am thankful to WESTGEN for supplying semen samples for my studies and the two research farms of the University of British Columbia for providing animals for my studies.

The completion of this dissertation would not be possible without the loving thoughts, encouragement and prayers of my parents, my wife Thiyahiny, in-laws, sisters, brother and other relatives.

FORWARD

This thesis is based on the following peer-reviewed scientific papers and conference presentations, which have been published or submitted for publication.

1. Giritharan G, Mohan M, Rajamahendran R. Effect of time of ovary removal on oocyte yield and in vitro embryo production in cattle. Proceedings of the Annual Meeting of Canadian Society of Animal Science 1998;335 (abst. 98T-42).

2. Giritharan G, Mohan M, Rajamahendran R. Maturation of bovine oocytes in straws: A method for transporting oocytes. Proceedings of the Annual Meeting of Canadian Society of Animal Science 1998;290 (abst. 98P-19).

3. Giritharan G, Dinn N, Rajamahendran R. In vitro embryo production from ovaries removed from culled dairy cows. Proceedings of the Annual Meeting of Canadian Society of Animal Science 1999;250 (abst. T-27).

4. Giritharan G, Afsani M, Lee G, Rajamahendran R. Effect of pre-treatment of sperm with progesterone and cholesterol on in vitro embryo production in cattle. Theriogenology 1999;51(1):318.

5. Giritharan G, Rajamahendran R. Maturation of bovine oocytes in straws: A method for long distance transport of oocytes for in vitro embryo production. (Abstract) Proceedings of the 14th International Congress on Animal Reproduction. July 2-6, 2000. Stockolm, Sweden.

Dedicated to My Parents and Wife
In Deep Appreciation of Their Love

CHAPTER I - GENERAL INTRODUCTION

The past decade witnessed considerable advancements in in-vitro oocyte maturation, fertilization and embryo culture, which has resulted in the large-scale production of bovine embryos. Attempts to accomplish in vitro fertilization with different species of animals were made prior to 1980, but reports of obtaining live offspring following embryo transfer (ET) were reported only in rabbits (Chang, 1959), mice (Wittingham, 1968), rats (Toyada and Chang, 1974), and man (Steptoe and Edwards, 1978). Although bovine ET techniques were widely practiced using in vivo produced embryos in the 1970s, attempts to use in vitro fertilization (IVF) to obtain live offspring following embryo transfer were not very successful. After the first report of in vitro fertilization of artificially matured bovine oocytes by Iritani and Niwa in 1977, Brackett (1982) reported the birth of the first live calf from in vitro fertilization of an ovulated oocyte in 1982. Hanada (1986) first reported production of calves from embryos obtained by in vitro oocyte maturation, fertilization, and in vivo embryo culture and Lu (1987) first reported production of calves from embryos obtained by totally in vitro procedures. Following these reports, various laboratories worldwide have started research into in-vitro maturation, fertilization and culture procedures to improve the success rate of in vitro embryo production. At present, most laboratories achieve more than 80% cleavage rate and 30-40% blastocyst formation rate, as well as a number of successful pregnancies and calves from in vitro produced frozen embryos (Saha et al., 1996; Pugh et al., 1998). Recently, in vitro embryo production technology has been used to produce pre-sexed, transgenic and cloned calves. Nuclear transfer and embryonic stem cell technologies, in

combination with in vitro embryo production technology, have also been used to produce identical copies of genetically valuable cows. These achievements indicate that in vitro embryo production technology is a very good tool for the enhancement of reproductive and genetic advances in cattle.

In the dairy industry, culled animals of high genetic merit are potential embryo donors. A means of extending in vitro embryo production technology to large and small pedigree farmers is by producing embryos from ovaries of genetically valuable culled cows before they are sent for slaughter. Survey reports have suggested that improving estrus detection could reduce the culling rate of cows indicating that the culled animals have good reproductive potential (Bascom and Young, 1998). These animals produce only five to six calves during their lifetime under the traditional breeding techniques. Quite a few reproductive technologies have been developed to increase the number of calves from these animals. Among these, in vivo embryo production technology, which involves super-ovulation, artificial insemination followed by embryo flushing and transfer is practical at the farm level (Calder and Rajamahendran, 1992). This technology however involves expensive hormonal treatments, increases calving interval of the valuable high-producing animals and thus, is not economically feasible. The other alternative is to remove the ovaries from culled cows before they are sent for slaughter and produce embryos in vitro.

Gametes play an important role in in-vitro embryo production technology. During the past, successful methods had been established for short and long term preservation of male gametes (Polge and Rowson, 1952). However, a successful method for short and long term preservation of female gametes has not been established yet. Techniques for the

cryopreservation of bovine embryos and semen have been well established during the past decades, but techniques for cryopreservation of bovine oocytes are still in the developmental stage. The oocyte is an extremely large, highly differentiated morphologically and functionally unique cell, and because of this, it reacts differently with cryoprotectants and chilling (Otoi *et al.,* 1997; Im *et al.,* 1998). Methods used for freezing and thawing affect the post thaw survival and developmental competence of the oocytes. A few studies reported that frozen oocytes can be used to establish pregnancy but the success rate is low (Lim et al., 1991; 1992; Otoi et al., 1992; Suzuki et al., 1996). There is also a high demand for mature and immature oocytes for advanced reproductive research. Therefore, exploring new methods for the preservation of bovine oocytes is important for commercial and research applications.

In the first experiment, I attempted to maximize embryo production from culled animals by using in-vitro techniques for the maturation, fertilization and culture of eggs. To increase embryo production from these cows, I tested the effect of ovary removal during the early part of the first follicular wave with and without FSH priming on oocyte yield and subsequent embryo development. The first follicular wave emerges immediately after estrus and is associated with a small surge release of FSH. The dominant follicle of this first follicular wave is selected 2 to 3 d post estrus. During this period, the developmental competence of all other follicles from the recruited pool is assumed to be good. Therefore, in my first experiment, I hypothesized that removal of ovaries during the early part of the follicular wave would yield more oocytes and embryos, and FSH priming at the time of the endogenous FSH surge could potentiate this effect in culled cows. In my second experiment, to establish a conventional maturation

system for successful transportation of bovine oocytes, I matured oocytes in straws in three different environments. In my third experiment, to establish a suitable cryopreservation method for bovine oocytes, I examined a cryoprotectant combination for vitrification, and one of the best available slow freezing techniques using oocytes in different stage of maturational.

CHAPTER 2 – REVIEW OF LITERATURE

2.1 - IN VITRO EMBRYO PRODUCTION

In vitro embryo production is an assisted reproductive technique in which oocytes are collected from ovaries, matured, fertilized and cultured in vitro to produce blastocysts or embryos. In this section, the evolution and achievements of each step of this technology from the beginning to present stage are discussed in detail.

2.1.1 - Collection of Oocytes

After the first report of in vitro fertilization of rabbit oocytes resulting in live offspring (Chang 1959), in vitro fertilization studies were conducted with bovine oocytes (Iritani and Niwa, 1977; Brackett *et al.,* 1982; Iritani et al., 1984; Hamano et al., 1992. An economical source of oocytes for these studies was from slaughterhouse ovaries. At that time, the main oocyte collection method was to aspirate the follicles using a needle and syringe. After the production of live bovine offspring by in vitro fertilization in 1982 (Brackett *et al.,* 1982), different recovery methods, such as needle aspiration, follicle dissection, and ovary slicing have been employed for collection of oocytes to maximize the yield from a single ovary for reproductive research. The aspiration method, in which the ovarian follicles were aspirated using an 18 gauge needle and a 10 ml syringe, resulted in approximately 8-12 oocytes per ovary with an acceptable quality of 45-50 percent (Katska and Smorak, 1984). Whereas, the follicle dissection method, in which

individual follicles were separated from ovaries and dissected in a petri dish, resulted in approximately 16-24 oocytes per ovary with an acceptable quality of 60-65 percent (Katska, 1984; Lonergan et. al., 1991). Xu *et al.* (1992a,b) obtained about 55-60 oocytes per ovary with 70 - 75 % acceptable quality oocytes by using the ovary slicing technique, in which ovaries were sliced in a petri dish, as described by Suss and Madison in 1983. Hamano and Kuwayama (1993) and Carolan *et al.* (1994) used the same technique and obtained approximately 32 and 44 oocytes per ovary, respectively. Another method of oocyte collection, a combination of aspiration and slicing, from slaughterhouse ovaries was described by Takagi *et al.* (1992) and they obtained about 32 oocytes per ovary. Strickland *et al.* (1976) reported a procedure in which they digested ovarian tissue by enzymes and obtained 221 oocytes per ovary but the viability of these oocytes was very low. The follicle aspiration method is preferred in most laboratories for processing large numbers of ovaries, when considering time and convenience, even though, the follicle dissection and ovary slicing techniques yield a high number of oocytes. Ovary slicing technique is preferred to process ovaries from one or two valuable animals.

High genetic merit animals have been used as a source for the in vivo collection of oocytes. Lambert *et al.* (1983, 1986) and Armstrong *et al.* (1991a, 1992) reported the use of laparoscopy through a paralumbar route for aspiration of follicles for in vitro fertilization and obtained a recovery rate of 72-79 percent. Armstrong (1991, 1991a, 1992, 1993) and Stubbings *et al.* (1993) showed that the response of the ovarian follicles of 3 to 9 week old heifer calves to superovulatory FSH treatment was very high. They also showed that oocytes recovered from these calves by laparoscopy were capable of producing embryos and calves after in vitro fertilization. In the laparoscopy technique,

most of the secondary oocytes could be aspirated from follicles, which were about to ovulate. Reichenbach *et al.* (1993, 1994) used this technique through the transvaginal route and collected oocytes for in vitro embryo production. After introduction of transvaginal ultrasound guided follicle aspiration (Callesen *et al.* 1987), superstimulation, ultrasound guided follicle aspiration, in vitro oocyte maturation, fertilization and embryo culture technique has been developed for the production of embryos for commercial and research purposes. However, the requirement for high-cost equipment and trained personal hindered common use of this technology (Kruip *et al.,* 1990, 1993; Maclellan *et al.,* 1998). Recently, this technology was used for the collection of oocytes from gonadotropin-treated prepubertal and pregnant animals for in vitro embryo production (Ryan *et al.,* 1990; Maclellan *et al.,* 1998). The animals used for this purpose were selected for specific characters from their pedigree information. Hence, selection of these animals is an expensive time consuming procedure. The other alternative source of animals for this purpose is culled cows.

2.1.2 – Selection of Oocytes

The quality of follicles determines the maturation and further developmental competence of oocytes. Quality of the oocytes varies with the size and nature of the vesicular follicles, as in cattle by the time follicles develop antria, the oocytes are still in the growing stage. (Thibault *et al.,* 1987; Tan and Lu, 1990; Gordon, 1994). Oocytes obtain meiotic competence when they are about 80% of the size of a full-grown oocyte (Thibault *et al.,* 1987). Pavlok *et al.* (1992, 1993) demonstrated that oocytes obtained

from follicles with a diameter of 1-2 mm showed reduced maturation and fertilization rates when compared to larger follicles. Lonergan *et al.* (1994) reported that more oocytes with many layers of cumulus cells and a resultant higher number of blastocysts, after in vitro maturation, fertilization and culture of oocytes, could be obtained from > 6 mm follicles compared to 2-6 mm follicles.

Reports showing different schemes for the identification of good quality follicles and oocytes have been published (Kruip and Dieleman, 1982; McNatty, 1984). Non-atretic follicles are characterized as having a uniformly bright appearance with very firm vascularization, a regular granulosa layer and no free floating particles in the follicular fluid (Kruip and Dieleman, 1982). Slightly atretic follicles are characterized as having a grayish appearance with the loss of some translucency and very small free-floating particles in the follicular fluid (Kruip and Dieleman, 1982). Atretic follicles are characterized as dull greyish with emptied blood vessels or clotted blood filled blood vessels (Kruip and Dieleman, 1982). Heavy atretic follicles are characterized as having a spotted dark appearance with a very dark cumulus mass (Kruip and Dieleman, 1982). McNatty (1984) also classified follicles as non-atretic, intermediate and atretic after observing them under the dissecting microscope. The presence of thecal cells in the membrana granulosa also reflects the early stage of atresia (Van Den Hurk, 1991). These differences in the surface appearance of large vesicular follicles reflect the quality of oocytes (Grimes and Ireland, 1985; 1987). Grimes *et al.* (1987) also demonstrated that the oocytes obtained from opaque follicles were most likely to be more atretic than those from clear or intermediate follicles. Leibfried and First (1979) showed that the stage of the estrous cycle could influence the quality of oocytes with the highest proportion of

degenerating oocytes being found in the follicular phase follicles. Follicular steroid levels can also be used to determine the quality of oocytes and the abnormal follicular steroid levels are associated with abnormal oocytes (Stubbings *et al*, 1990). Morphological characteristics of the follicles and bovine follicular fluid progesterone (P_4) concentrations determine the quality of oocytes (Hazeleger *et al.,* 1993).

Based on the cumulus investment and morphological appearance of the cytoplasm, oocytes can be selected for maturation and further development in vitro. Leibfried and First (1979) first reported a scheme for the selection of bovine oocytes based on morphological features. Since their report, various reports have been published on selection of oocytes based on quality of the cytoplasm, and compactness and quantity of the surrounding cumulus cells (Suss and Madison, 1983; Katska and Smorag, 1984; Shioya *et al.,* 1988; Tan *et al.,* 1988; Loos *et al.,* 1989, 1991; Younis *et al.,* 1989; Kim and Park, 1990; Yang and Lu, 1990; Dominko and First, 1991; Hazeleger and Stubbings, 1992; Madison *et al.,* 1992). Loos *et al.* (1989) classified oocytes into four categories and studied maturational competence. Category 1 oocytes had compact multi-layered cumulus investment with homogeneous ooplasm and the total cumulus oocyte complex had a light and transparent appearance. Category 2 oocytes had compact multi-layered cumulus investment with homogeneous ooplasm and the total cumulus oocyte complex had a slightly darker and less transparent appearance with a darker zone around the oocyte. Category 3 oocytes had a less compact cumulus investment and an irregular ooplasm with dark clusters, and the total cumulus oocyte complex had darker appearance than categories 1 and 2. Category 4 oocytes had expanded and scattered cumulus cells in dark clumps with a jelly matrix, and an irregular ooplasm with dark clusters, and the total

cumulus oocyte complex had darker and irregular appearance. Categories 1 to 3 had similar developmental competence (Loos *et al.,* 1989). Laurincik *et al.* (1992) classified oocytes into two groups, as those with a dark rim of corona cells around the zona pellucida and those with the same density as the cumulus cell mass, and compared maturation rate of these groups. They concluded that the oocytes with a dark rim of corona cells matured at a higher rate than the other group of oocytes. Lonergan *et al.* (1994) classified oocytes into five groups and showed that a higher number of multi-layered oocytes could be obtained from > 6 mm follicles and higher number of blastocysts could be obtained from these oocytes after in vitro fertilization and culture. Based on these studies and considering the time factor, different laboratories have their own procedures for the selection of oocytes but the methods described by Lonergan *et al.* and Loos *et al.* are the most commonly used.

2.1.3 - In Vitro Maturation

Cytology of bovine oocyte maturation was well studied during the past decades (Sreenan, 1970; Jagiello *et al.,* 1974; Motlik *et al.,* 1986; Hyttel *et al.,* 1986; Sirard *et al.,* 1989; Monaghan *et al.,* 1993). During the oocyte maturation process, bovine oocytes, which are arrested at prophase of the first meiotic division, undergo reduction division and are again arrested at metaphase I. Oocytes complete their first meiotic division by germinal vesicle break-down and extrusion of the first polar body during the final stage of their maturation. These oocytes then resume their second meiotic division, are arrested at metaphase II and complete the second meiotic division at fertilization after obtaining

stimulus from sperm. The maturation process of oocytes involves not only nuclear maturation, but also cytoplasmic maturation with several interactions of cell cycle molecules and their target substrates (Gordon, 1994).

From 1960 onwards, attempts have been made to mature bovine oocytes in vitro. Rush *et al.* (1973) tried to culture intact follicles to obtain mature oocytes but no evidence of oocyte maturation was reported. In contrast to their findings, Moor and Trounson (1977) reported that about 40-50 percent of sheep oocytes could be matured through intact follicle culture techniques. This finding was supported by the study of Fukuii *et al.* (1987) in which extra- and intra- follicular maturation were compared and it was concluded that intra-follicular maturation was most suitable for in vitro maturation of oocytes. Although intra-follicular maturation of bovine oocytes seems more suitable than conventional in vitro maturation technique, it is time consuming and requires considerable skill. Hence, intra-follicular maturation is not useful for the production of embryos in large numbers and most laboratories use conventional extra-follicular maturation culture systems for the production of embryos in vitro.

It is well known that the culture conditions employed for conventional in vitro maturation methods of bovine oocytes influence subsequent in vitro fertilization and embryo development (Ball et al., 1983; Brackett et al., 1989; Chung and Seidel, 1993; Angelika et al., 1997). The culture media used for in vitro maturation of bovine oocytes is broadly divided into simple and complex media. Simple media are usually bicarbonate-buffered systems containing basic physiological saline with pyruvate, lactate and glucose. The simple media are generally supplemented with serum or albumin with trace amounts of antibiotics. Complex media contain, in addition to the basic components of the simple

media, amino acids, vitamins, purines, and other substances, mainly at levels at which they are found in serum (Gordon 1994). Rose and Bavister (1992) examined seven commercially available culture media for in vitro maturation and reported that the media used for in vitro maturation significantly affect subsequent embryo development. Among the available complex media tissue culture medium 199 (TCM199) is most commonly used for in vitro maturation (Suzuki *et al.,* 1996). TCM 199 consists of pyruvate, lactate, amino acids, vitamins, purines, and other substances as found in serum, Earle's salts with buffer *N*-(2hydroxethyl)-piperazine-*N'*-(ethanesulphonic acid) (HEPES) and sodium bicarbonate (Gordon, 1994). However, some researchers concluded that the Ham's F10 medium was superior under their culture conditions by comparing complex Ham's F10 medium with TCM199 (Xu *et al.,* 1987, 1992; Hawk and Wall, 1993).

Addition of serum, hormones, growth factors, and specific chemicals to culture media has been shown to improve in vitro maturation of oocytes. Various forms of bovine serum, such as fetal calf serum, estrus cow serum and superovulated cow serum have been employed as a protein source in in vitro culture media. Lu and Gordon (1987) and Lu *et al.* (1987) reported that estrus cow serum was superior to fetal calf serum for this purpose. Chung and Seidel (1993) reported that the addition of 0.1 mM ethylenediaminetetraacetic acid (EDTA) and supplements to the in vitro maturation media TCM199 improved the maturation and subsequent embryo development. Although some studies (Lu *et al.,* 1987, 1988; Trounson *et al.,* 1994) showed that addition of hormones or growth factors had no effect on oocyte maturation, addition of gonadotropins and growth factors to in vitro maturation media has been a common practice as other studies showed beneficial effects. Brackett *et al.* (1989), and Zuelke and

Brackett (1990) demonstrated that the addition of luteinizing hormone (LH) to in vitro maturation media enhanced oocyte quality and subsequent embryo development. Although, beneficial effects of follicle stimulating hormone (FSH) on in vitro maturation of oocytes has not been shown, addition of FSH to maturation media has been practiced in most laboratories (Fukushima and Fukui, 1985; Fukui and Ono, 1988; Sirard *et al.,* 1989; Saeki *et al.,* 1990). Recently, Park *et al.* (1997) reported that addition of epidermal growth factor to chemically defined in vitro maturation media increased nuclear and cytoplasmic maturation of oocytes. Angelika *et al.* (1997) showed that addition of activin A and inhibin A to hormone free and serum free maturation media markedly increased subsequent in vitro embryo development. These findings revealed that various growth factors support the maturation process and those growth factors are present in the serum supplement or produced by co-cultured cells.

Most in vitro embryo production studies indicate that a bicarbonate/CO_2 buffered environment is ideal for maturation of bovine oocytes (Staigmiller *et al.,* 1984; Crozet *et al.,* 1991; Moore *et al.,* 1993). Azambuja *et al.* (1993) studied the effects of a 5% CO_2 air gas phase and a 5% CO_2, 5% O_2, 90% N_2 gas phase on maturation of bovine oocytes and concluded that the conventional 5% CO_2 air gas phase was most suitable to obtain optimal embryo development. Pinyopummintr and Bavister (1994) also examined the effects of various gas phases and concluded that a low oxygen concentration (5-10%) was detrimental for both maturation and fertilization of bovine oocytes. However, it has been shown that the oocytes can also be matured in non-5%CO_2 environment resulting in viable embryos after in vitro fertilization and culture (Westhusin *et al.,* 1992; and Byrd *et al.,* 1997).

Provision of optimum temperature for in vitro maturation of bovine oocytes is important to obtain optimum maturation and subsequent embryo development. Until 1980, studies in relation to bovine in vitro maturation had been done at a temperature of 37 °C. Lenz *et al.* first reported the effect of culture temperature on in vitro maturation in 1983. They showed that maturation was not affected at a range of temperatures between 35 °C and 39 °C. Their findings were supported by the studies of Katska and Smarog (1985), and Morstin and Katska (1986). However, Wang *et al.* (1991) showed that the optimum temperature for in vitro maturation of bovine oocytes is 38-39 °C.

Studies have been conducted in the past to evaluate the timing of nuclear events during the maturation process. Usually the time required for a primary oocyte to reach the secondary oocyte stage, after expulsion of first polar body, is 18-24 h after initiation of maturation process (Sreenan, 1970; Jagiello *et al.,* 1974; Motlik *et al.,* 1986; Hyttel *et al.,* 1986; Sirard *et al.,* 1989; Monaghan *et al.,* 1993). The study conducted by Sirard *et al.* (1989) on the timing of nuclear events revealed that germinal vesicle, germinal vesicle breakdown, chromatin condensation, metaphase I, anaphase I, telophase I and metaphase II were evident at 0-6.6 h, 6.6-8.0 h, 8.0-10.3 h, 10.3-15.4 h, 15.4-16.6 h, 16.6-18.0 h and 18-24 h, respectively. Other studies showed that timing of oocyte maturation depends on the thickness of the cumulus investment (Spiropoulos and Long, 1989). Oocytes with less cumulus investment progress to metaphase II faster than compact cumulus oocyte complexes. These findings show that maturation of immature bovine oocytes for 18-24 h yields optimum maturation and further development after in vitro fertilization and culture.

At present, most laboratories have their own in vitro maturation protocols. The protocol most commonly used is maturation of bovine oocytes in TCM 199 supplemented

with gonadotropin and 5-10% serum in an environment containing 5% CO_2 in air gas phase at 38-39 °C for 18-24 h Suzuki et al., 1996, Saha et al., 1996; Bordignon et al., 1997; Krisher and Bovister, 1998).

2.1.4 - In Vitro Fertilization

Fertilization, in which male gametes fuse with female gametes and initiate a series of cell divisions to form new individuals, is a complex process. Success in this process depends on provision of optimum conditions for male and female gametes. Sperm, after being deposited in the female reproductive tract, undergoes certain maturational changes (capacitation) during its travel before arriving at the oviduct and being able to fertilize the ovum (Chang, 1951). The capacitation process is a major barrier for successful fertilization in vitro (Chang, 1951). To overcome this problem, several methods to obtain capacitated sperm were explored in fertilization studies in the past. After first report on the birth of live offspring by in vitro fertilization using in vivo capacitated sperm and embryo transfer in rabbit (Chang, 1959), attempts were made to implement this technique in different species of animals. Brackett and Oliphant (1975) reported an in vitro capacitation procedure, in which rabbit spermatozoa were capacitated by altering sperm plasma membranes using hyper ionic solutions during in vitro fertilization. Iritani and Niwa (1977) first showed that bovine spermatozoa could be capacitated using rabbit uteri and cattle oviducts. After this report, attempts to capacitate bovine sperm in vitro were made by researchers. The first successful bovine in vitro fertilization of ovulated oocytes using in vitro capacitated sperm in high ionic strength solutions resulting in the birth of

live offspring was reported by Brackett *et al.* (1982). Bondioli *et al.* (1983) also reported successful fertilization of bovine oocytes by in vitro capacitated spermatozoa. Although the capacitation of bovine sperm can be induced by hyper-ionic solutions, it can not be achieved well unless specific capacitation agents and methods are used (Ball *et al.,* 1983). Capacitating agents, such as heparin (Parrish *et al.,* 1984; Lancaster, 1990), calcium ionophore (A23187) (Jiang *et al.,* 1991; Jiang, 1992), have been used for the capacitation of bull sperm in the past, although heparin is most commonly used for in vitro embryo production protocols.

Preparation of sperm has been shown to be significant factor for successful in vitro fertilization. Sperm should be washed properly to remove either seminal plasma, if they are fresh, or constituents of the cryopreservation medium, if they were frozen. Lu *et al.* (1987, 1990), and Ijaz and Hunter (1989) reported a rapid washing method in which sperm cells were centrifuged twice in a calcium free TALP medium at 500 *g* for 10 min, before allowing them to swim up to obtain the most viable sperm. Some researchers suggest that after washing, sperm cells should be kept in capacitation media at 25 °C for 4-8 h for optimum fertilization and subsequent embryo production (Chen *et al.,* 1992; Fair, 1992). In cattle in vitro fertilization, concentration of sperm varies from 0.5 to 5 million per ml and the sperm : oocyte ratio varies from 10000 to 20000:1 (Brackett *et al.,* 1982; Bousquet *et al.,* 1984; Lambert *et al.,* 1986; Parrish *et al.,* 1986; Lu *et al.,* 1987; Ling and Lu, 1990).

2.1.5 - In Vitro Culture

In vitro culture of bovine embryos requires a culture system, which mimics the environment of the oviduct so that the early embryo can develop to the blastocyst stage. Previously, most in vitro embryo culture work was done with rabbit and mouse embryos. In the 1960s, researchers tried to use oviducts from various animals as in vivo culture system for bovine embryos. Sreenan and Scanlon (1968) first reported the successful culture of cattle embryos up to the blastocyst stage outside the cow. Rabbit oviducts were commonly used as a temporary incubator to store cattle embryos and produced normal conceptus after embryo transfer to recipient animals (Gordon, 1975 & 1976; Boland, 1984; Hanada, 1985; Sirard *et al.,* 1985; Lambert *et al.,* 1986). Fehilly *et al.* (1984) reported that sheep oviducts provided a suitable environment, not only to sheep embryos, but also to those from other species of animals. Parrish *et al.* (1986), Eyestone *et al.* (1987) and Lu *et al.* (1987, 1987a, 1988) also reported that ovine oviducts could be used to culture bovine embryos up to the blastocyst stage. It was assumed that sheep oviducts were more suitable than rabbit oviducts for in vivo culture of bovine embryos due to species relationships. Comparison of these two culture systems revealed that similar results could be obtained from these two culture systems (Lutterbach *et al.,* 1987; Fukui *et al.,* 1989; Westhusin *et al.,* 1989). Sheep oviducts are preferred over rabbit oviducts due to their relative size and relative convenience in controlling estrous cycle of sheep. Some other reports stated that the cow oviducts could also be used as in vivo culture system (Newcomb, 1978; Xu *et al.,* 1987). Blakewood *et al.* (1988, 1989, 1989a, 1990) cultured mice, goat and cattle embryos in the amniotic cavity of the four-day old chicken

eggs and concluded that rates of embryo development were comparable to the development in vivo. This technique was tedious and unsuited for the large-scale culture of embryos because it was difficult to keep these embryos in the amniotic cavity of an egg for more than 3 days. To overcome this problem, Blakewood and Zhang (1993) tried to culture mouse embryos in extracted amniotic fluid. Their result revealed that the rate of embryo development was lower than that of in situ amniotic fluid culture. Isolated mouse oviducts were used as an in vivo culture system for bovine embryos by Sharif *et al.* (1991, 1992 and 1993) but there was no advantage observed over other conventional in vitro culture systems.

The major objective of the conventional culture system is to provide an optimum culture environment for the development of embryos and to obtain a similar number of embryos as in in-vivo condition. Limited development of embryos was observed when culturing embryos in conventional tissue culture media during early 1950s. Tervit *et al.* (1972) first formulated a synthetic medium based on the biochemical analysis of sheep oviducts, named synthetic oviductal fluid, and successfully cultured one to eight cell cattle embryos to morula and blastocyst stages using 5% oxygen in an air-gas phase environment. Since then, several laboratories examined different culture media and gas phases to obtain maximum success in culturing embryos in vitro (Wright *et al.,* 1976a,b; Thompson *et al.,* 1989; Fukui *et al.,* 1991, Rorie 1991a,b; Fontes 1991, 1992; Takahashi and first, 1992, 1993; McLaughlin *et al.,* 1992; Trounson *et al.,* 1994). Fukui *et al.* (1991) developed a culture system in which they cultured bovine embryos in synthetic oviductal fluid medium supplemented with fetal calf serum or human serum in a 5% oxygen-air phase environment. Many culture media formulations used to culture early embryos are

simple and contain Kreb's salt solution supplemented with energy sources such as pyruvate, glucose and lactate, and a protein source such as fetal calf serum or bovine serum albumin. The complex media most commonly used for the culture of early embryos are TCM199 and Ham's F–10. Shamsuddin *et al.* (1993a,b) successfully grew in vitro matured and fertilized embryos up to blastocyst stage in TCM199 without somatic cells. Some studies suggested that TCM199 was preferable to Ham's F-10 (Lu *et al.*, 1988). Semple *et al.* (1993) compared TCM199 and Menezo's B medium for the culture of bovine embryos and reported that both gave comparable blastocyst yields.

In most of the above studies, very few embryos passed the 8-cell block. To overcome this problem, most laboratories tried to coculture embryos with different kinds of cell layers. Rorie *et al.* (1991a,b) showed that culturing bovine embryos in synthetic oviductal fluid medium supplemented with fetal calf serum and oviductal cell coculture would improve their morphological quality. Mermillod *et al.* (1991) compared rabbit and bovine oviductal epithelial cell monolayers for the coculture of bovine early embryos and reported that although coculture with a rabbit oviductal epithelial cell monolayer supported embryo growth, coculture with a bovine oviductal epithelial cell monolayer gave a higher blastocyst yield. Eyestone and First (1989) showed that embryos obtained from in vivo matured oocytes gave similar blastocyst yields when cultured with a bovine oviductal epithelial monolayer or sheep oviduct. McCaffrey and Sreenan (1991) reported that a bovine oviductal epithelial cell monolayer culture system was much more effective in the development of in vivo produced embryos than that of in vitro matured and fertilized embryos. Lu *et al.* (1987a, 1988) and Wang *et al.* (1989) showed that TCM199 was superior to Ham's F-10 medium with a bovine epithelial cell monolayer and estrous

cow serum for the development of bovine early embryos. Xu *et al.* (1992) suggested that Menezo's B2 medium with 10% estrous cow serum was superior to all other media tested for the coculture of bovine in vitro matured and fertilized embryos with bovine oviduct epithelial cell monolayer. Fontes *et al.* (1993) and Lim *et al.* (1996a) reported that frozen oviductal cells could be successfully used to culture bovine embryos up to blastocyst stage. Lu *et al.* (1988) observed that some bovine in vitro matured and fertilized embryos, which had some cumulus cells attached, and which were cultured in TCM199 passed through the 8-cell block. They suggested that this result might be due to the activity of cumulus cells. Goto *et al.* (1988a,b), Fukuda *et al.* (1990), Kajihara *et al.* (1990), Nakao and Nakatsuji (1990), and Lim *et al.* (1996b) cocultured in vitro matured and fertilized bovine embryos with cumulus cells to the blastocyst stage and obtained successful pregnancies. Some studies suggested that granulosa cell monolayers could be used as an alternative to cumulus cell monolayers for the coculture of bovine embryos to get similar blastocyst yield (Jiang *et al.,* 1990; Ling and Lu, 1990; Tan and Lu, 1990; Wang *et al.,* 1990). Scodras *et al.* (1991) cultured bovine embryos with different kinds of cell lines and reported that cell lines from a variety of tissues and species would support bovine early embryo development. This finding was also supported by the study of Goto *et al.* (1992) in which they compared different cell lines from embryos of different species and reported that all cell lines equally supported bovine embryo development. Inzen *et al.* (1994) showed that bovine embryos could be successfully cultured up to the blastocyst stage with buffalo rat liver cells. Palma *et al.* (1992), Donnay *et al.* (1995) and O'Doherty *et al.* (1997) showed that culturing bovine embryos in groups had a beneficial effect on embryo development. Jiang *et al.* (1991) cocultured bovine embryos with monolayers of

granulosa cells, oviductal cells, uterine cells or combination of these cells and concluded that, except for uterine cell monolayer, all other cell monolayers gave similar embryo yield. Because cumulus cells can be readily available as part of routine bovine in vitro maturation and fertilization procedures, most of the laboratories are using cumulus cells to coculture bovine embryos.

Attempts were made to standardize coculture systems by culturing embryos in pre-conditioned media with different types of cells. Eyestone and First (1989) successfully cultured bovine embryos to blastocyst stage in pre-conditioned media with bovine oviductal epithelial cells. They also reported that media conditioned with bovine oviductal cells collected from different stages of estrous cycle had no effect on embryo development. In contrast to their findings, Harper *et al.* (1989) demonstrated that higher rates of embryo development could be obtained by culturing embryos in conditioned media prepared using oviductal cells obtained around the time of estrus. Mermillod *et al.* (1993) cultured embryos in a serum free conditioned media and reported that some embryotropic factors, which were present in the conditioned media, potentiated the development of early embryos. Inzen *et al.* (1993) reported that bovine embryos could be cultured in Buffalo Rat cell culture conditioned media as effectively as in Buffalo Rat cell coculture. Some studies suggested that conditioned media prepared from actively dividing cells and cell suspensions could promote embryo development better than cell monolayers (Vergos *et al.,* 1991; Xu *et al.,* 1992a,b,c). Maeda *et al.* (1996) showed that bovine embryos could be cultured in exogenous protein- and amino acid-free chemically defined human tubal fluid media conditioned with granulosa cells. Kobayashi *et al.* (1991) cultured bovine embryos in conditioned chemically defined media obtained from

oviductal and granulosa cell cultures and concluded that both these cell types produced some soluble growth promoting factors, which potentiated early embryonic growth. These studies suggested that conditioned media obtained from various cell cultures could be used for the successful culture of bovine embryos.

Much evidence has been published on the regulation of early embryonic development by hormones and growth factors. Gandolfi *et al.* (1991) detected and characterized growth factors in bovine oviductal secretion. Watson *et al.* (1992) showed that growth factor ligand and receptor genes were expressed during bovine early embryonic development. Host-Hansen *et al.* (1993) reported that cultured bovine oviductal epithelial cells could express and secrete growth factors. After these findings, different laboratories explored effects of growth factors on pre-implantation bovine embryo development (Kaye *et al.,* 1992; Keefer, 1992; Larson *et al.,* 1992a,b; Flood *et al.,* 1993; Heyner *et al.,* 1993; White *et al.,* 1994; Lee and Fukui, 1995; Lim and Hansel, 1996). Watson *et al.* (1992) showed that transcripts for insulin like growth factor-II (IGF-II) and receptors for insulin, insulin like growth factor-I (IGF-I) and IGF-II were detectable in bovine embryos. Simmen *et al.* (1993) also reported that IGF-I could improve the development of embryos. Larson *et al.* (1992b) reported that platelet-derived growth factor (PDGF) could stimulate the development of bovine embryos during the fourth cell cycle. Thibodeaux *et al.* (1993) also reported that PDGF provided a developmental stimulus similar to bovine oviductal epithelial cells for bovine embryos at the fourth cell cycle. Eckert and Niemann (1993) showed the enhancement of in vitro derived bovine embryo development by addition of PDGF to the culture media at various stages of embryo development. Yang *et al.* (1993a,b) compared the effect of different

growth factors on in vitro development of bovine embryos and concluded that most of the beneficial effects on development to the blastocyst stage were from PDGF when all other growth factors were compared. Thibodeaux *et al.* (1995) showed that the beneficial effects of incubating bovine embryos in groups were due to action of PDGF. Eckert and Niemann (1996) examined the effect of adding PDGF and estrous cow serum to protein-free culture media on the development of bovine embryos and concluded that PDGF increased the embryo development as effectively as estrous cow serum. Flood and Bunch (1992) and Flood *et al.* (1993) showed that the addition of epidermal growth factor (EGF) to in vitro culture media increased bovine embryo development to the blastocyst stage. Keefer (1992) examined the effect of addition of EGF to simple culture media on bovine in vitro embryo development and concluded that it increased the hatching rate of bovine blastocysts. Yang *et al.* (1993a,b) also showed that the addition of EGF to culture media would improve the development of bovine embryos to blastocyst stage. Larson *et al.* (1992a) investigated the effect of adding transforming growth factor β and basic fibroblast growth factor on in vitro development of bovine embryos and reported that both synergistically promoted early embryonic development. Lee and Fukui (1995) examined the effect of growth factors in a defined culture medium on development of bovine embryos matured and fertilized in vitro and found that fibroblast growth factor (FGF), EGF or both would increase development of bovine embryos to morula and blastocyst stages. They also suggested that FGF and EGF might act synergistically on bovine development in vitro. Fukui and Matsuyama (1994) showed that addition of human leukemia inhibitory factor to the culture media could increase the development of bovine embryos in vitro. De Moraes and Hansen (1997) investigated the effects of adding

granulocyte-macrophage colony stimulating factor to culture media on the development of in vitro produced bovine embryos and reported that addition of this factor could promote in vitro development of embryos. Koji Yoshioka *et al.* (1998) examined the effects of addition of activin-A and follistatin to chemically defined culture media on the development of bovine embryos in vitro and found that activin-A increased and follistatin decreased the rate of embryo development. They suggested that activin-A and follistatin were involved in the regulation of bovine embryo development. Most of the above studies were done with chemically defined media whereas conditioned media or coculturing in a complex media with estrous cow serum would give similar blastocyst yields, which suggested that coculturing cells might produce or estrous cow serum might contain most of these growth factors and hormones. Based on these studies, different laboratories use chemically defined media with different growth factors for in vitro culture in experiments on embryo metabolism and coculturing or conditioned media with estrous cow serum for in vitro culture for other embryo works.

It has been well understood that culturing embryos at optimal temperature would be important for successful embryo development in vitro. Wang *et al.*(1991) cultured embryos at a series of different temperatures from 36 °C to 40 °C and concluded that culturing embryos between 37 °C to 39 °C gives optimum embryo yield. Alfonso and Hunter (1992a,b) also examined the effect of temperature on development of bovine embryos and found that a higher number of embryos cleaved in 37 °C culture than in 39 °C culture. Based on the above studies, bovine embryos are cultured between 38° C and 39° C in most of laboratories.

2.2 - DEVELOPMENT OF OOCYTES AND FOLLICLES

The cow is a monotocus and polyestrus animal. During the estrous cycle, a series of physiological events such as recruitment and selection of follicles, ovulation of a single dominant follicle, and formation and regression of a corpus luteum occur in a cyclic manner. Also, during these cyclic events, the oocyte of each ovulatory follicle, which is arrested in prophase of the first meiotic division, starts its growth, completes its first meiotic division and is arrested again at the metaphase of the second meiotic division. These events are controlled by various reproductive hormones secreted by the hypothalamus, pituitary gland and ovaries and can be altered by exogenous treatment of these hormones. The development of bovine oocytes, follicular dynamics and the associated hormonal changes are reviewed in detail in the following part of the chapter.

2.2.1 - Development of Oocytes and Follicles

Development of oocytes begins with the migration of primodial germ cells from the yolk sac to the undifferentiated gonads of the developing embryo (Baker, 1971). When primodial germ cells arrive, the epithelium of the undifferentiated gonad proliferates actively and becomes thick forming the genital cords. After this, the germ cells undergo a series of mitotic divisions before entering into meiosis and increasing in number to reach approximately 2 million cells per animal. About 95% of these germ cells have degenerated at the time of birth (Erickson, 1966). After entering meiosis, the oogonia are arrested at the diplotene stage to form a dictyate stage primary oocyte, which

is surrounded by a single layer of somatic cells from the gonads and is called a primodial follicle (Gordon, 1994).

The primodial follicles, which occupy the periphery of the ovarian cortex, constantly enter into growth phase, and are separated from each other by cells of the ovarian stroma, bundles of collagen and reticular fibers, and blood vessels. In the growth phase, primodial follicles increase in size by active division of the surrounding cells of the oocytes. Although, the nuclei of the primodial follicle oocytes are quiescent in division, they are active in the production of RNA and proteins. Hence, the primary oocytes also increase in size.

During the growth phase of the oocytes, marked changes occur in number and structure of the organelles. The chromosomes in the nucleus or germinal vesicle of the growing oocytes remain as highly diffuse haploids while the germinal vesicle increases in size (Gordon, 1994). Vacuolated fibrillogranular nucleoli progressively change their structure to contain a large vacuole at the center surrounded by fibrillogranular material, which indicates active RNA synthesis (Motlik and Fulka, 1986, Gordon, 1994). When the growing oocytes reach their full size, the nucleoli become progressively formed by a compact electron-dense fibrillar material and fibrillogranular vacuolated areas localized at the nucleolar periphery (Motlik and Fulka, 1986, Gordon, 1994). Nucleolar compaction is associated with the formation of few small nucleoli each containing a large fibrillar center, through fragmentation of one or two large nucleoli. (Crozet *et al.,* 1986; Crozet, 1989). Mitochondria, which are in close association with the smooth endoplasmic reticulum, increase in number, change their structure from elongated to round or oval, became highly vacuolated and cluster intensively in the cortical regions of the oocyte

(Fleming and Saacke, 1972; Kruip *et al.,* 1983). At the end of maturation, these clustered mitochondria are present in the central region of the oocyte (Fleming and Saacke, 1972; Kruip *et al.,* 1983). The presence of numerous, swollen, stacked lamellae that are associated with several vacuoles, granules, coated vesicles and lipid vesicles in the Golgi complexes indicates that the Golgi complexes are actively producing secretary products and cortical granules (Gordon, 1994). Cortical granules are small, spherical, membrane bound lysosome like organelles, which have an active role in the prevention of polyspermic fertilization. They are not evident in the primordial follicle and formed with the growth and differentiation of the oocytes (Kruip *et al.,* 1983; Cran, 1987, 1989). An increase in the number of ribosomes, which are present in the polysomes, represents a high rate of protein synthesis during oocyte growth (Gordon, 1994). Cytoplasmic lattices, which have unknown function, also significantly increase in number throughout the growth period of oocytes (Gordon, 1994). While the primary oocytes increase in size, a denser and thicker mesh work of interconnected filaments surround the oocytes to form a thick extra cellular coat, called the zona pellucida, which separates oocytes from surrounding granulosa cells. Although, zona pellucida separates oocytes and granulosa cells, contact between these cells and the oocytes are established via junction complexes of oocyte microvilli and granulosa cell extensions (Baker, 1971, Gordon, 1994). These contacts and junction complexes are important for the growth and final maturation of the oocytes. While follicles increase in diameter from 0.12 to 0.16 mm, small covitations appear among granulosa cells (Marrion *et al.,* 1968; Dufour and Roy, 1985; Lussier *et al* 1987). However, in some studies, it has been shown that the cavitations first appear in bovine follicles in a diameter range of 0.115 mm to 0.280 mm (Monniaux *et al.,* 1983).

These cavities fuse together to form a large cavity, called the follicular antrum (Marrion *et al.,* 1968; Monniaux *et al.,* 1983; Dufour and Roy, 1985; Lussier *et al* 1987). Follicles with an antrum are called antral or vesicular follicles and are present in the ovaries from birth to old age of the animals (Erickson, 1966). The oocyte of antral follicles continues to grow until the follicles reach a diameter of 2-3 mm (Gordon, 1990). At the end of this growth phase, bovine oocytes obtain a diameter of about 130 μm and meiotic competence for further maturation (Gordon, 1990). It is also shown that vesicular follicles are present in the calf's ovary before birth (Mariana *et al.,* 1991). The factors involved in the regulation of preantral follicular growth and recruitment are not well known. Although, it is well documented, that preantral follicular growth is not regulated by circulating gonadotropins (Ryle, 1972; Hirshfield, 1985; Cain *et al.,* 1995; Braw-Tal and Yossefi, 1997), the involvement of FSH and high local concentrations of estrogen has been shown by some laboratories (Wang and Greenwald, 1993; Hulshof *et al.,* 1995; Wandji *et al.,* 1996). At present, an enormous number of reports are available on the pattern of follicular growth involving follicular recruitment, selection, atresia and dominance, and associated hormonal changes.

2.2.2 - Bovine Ovarian Follicular Dynamics and Associated Hormonal Changes

Since 1960, it has been proposed that two waves of follicular activity are present in cattle (Rajakoski *et al.,* 1960). Results of a study conducted by Matton *et al.* (1981) are consistent with the two-wave hypothesis. However, in 1983, Ireland *et al.* showed that some animals might show three follicular waves as well. Each follicular wave resulted in

a dominant follicle and whether there are two or three follicular waves, follicles develop rapidly and undergo atresia until an ovulatory sized follicle appeared (Ireland *et al.,* 1983; Spicer *et al.,* 1986). After the invention of transrectal ultrasound imaging, various reports have been published on follicular dynamics of cattle (Pierson *et al.,* 1988; Fortune *et al.,* 1988; Savio *et al.,* 1988; Sirois *et al.,* 1988; Ginther *et al.,* 1989). The number of follicular waves, whether two or three, is determined by dietary intake (Murphy *et al.,* 1991), parity and lactation status (Lucy *et al.,* 1992) of the animal. In animals with a three-wave cycle, the first follicular wave emerges just before the day of ovulation, the second wave emerges ten days after ovulation and the third wave emerges 16 days after ovulation. In each follicular wave, one follicle becomes dominant and the others become subordinate. In each estrous cycle, the dominant follicle of the last follicular wave ovulates and the dominant follicles of the other follicular waves undergo atresia (Ginther *et al.,* 1989 & 1996, Driancourt *et al.,* 1991; Campbell *et al.,* 1995). The deviation in growth of newly recruited follicles toward development of a dominant follicle occurs on day two of each follicular wave and, thereafter, the developing dominant follicle suppresses growth of other follicles (Adams *et al.,* 1993; Ginther *et al.,* 1996).

Hormones and growth factors, produced by the hypothalamus, pituitary gland and ovaries, regulate the ovarian follicular dynamics. The most common hormones involved in this process are GnRH, FSH, LH, P_4 and E_2, and the most common growth factors are inhibin, activin and insulin like growth factors (IGFs). The major hypothalamic regulator of bovine reproductive function is GnRH which regulates the production and release of gonadotropins (FSH and LH). GnRH secretion is regulated by ovarian steroids and endorphins and normal gonadotropin secretion requires the pulsatile secretion of GnRH

because continuous exposure to GnRH causes a loss of GnRH receptor recycling in the anterior pituitary, which leads to suppression of gonadotropin release (Strauss III and Steinkampf, 1995). FSH and LH are the key-regulators of bovine follicular dynamics and are secreted in surge and pulsatile patterns, respectively. Their secretion is regulated by estrogen, progesterone and inhibin produced by the ovaries through direct action on the pituitary or indirect action on the hypothalamus on the secretion of GnRH (Findlay and Clarke, 1987). It has been shown that a follicular wave emerges at the basal concentration of gonadotropins every 7-9 d and the number of follicular waves are determined by time of luteolysis (Savio *et al.,* 1990; Taylor and Rajamahendran, 1991). Each follicular wave is associated with a small FSH surge (Hamilton *et al.,* 1992; Sunderland *et al.,* 1994; Gong *et al.,* 1995; Bodensteiner *et al.,* 1996), therefore, altering FSH and LH secretion by various exogenous hormone treatments or exogenous FSH and LH treatment alters the follicular dynamics in cattle.

2.3 - PRESERVATION OF EMBRYOS AND OOCYTES

In livestock farming, the effective exploitation of embryo transfer technology depends on establishment of effective techniques for the preservation of embryos and oocytes. The major purpose of preserving embryos is to keep them, for a short or long period, in a suspended state from which they may regain their developmental capacity when optimal conditions are provided. Various attempts were made to preserve bovine embryos for a short period due to time requirement from embryo collection to transfer or cryopreservation in the embryo transfer industry. It has been shown that embryos kept in

phosphate buffered saline (PBS), supplemented with serum, for an extended period at room temperature had a reduced developmental potential (Wright, 1986; Aoyagi *et al.*, 1990). Different media ranging from complex such as TCM199 and Ham's F-10 to simple media such as PBS supplemented with serum were used in the short-term preservation of embryos prior to transfer or cryopreservation. Rajamahendran *et al.* (1985), Smith *et al.* (1986) and Hasler *et al.* (1987) concluded that Ham's F-10 media was the media of choice for short-term preservation of embryos in terms of growth, survival and hatchability. The problem of decreased developmental capacity of bovine embryos, which were kept in a non-nutrient media for extended periods at room temperature or a higher temperature, can be overcome by preserving them at refrigeration temperature (Trounson *et al.*, 1976a,b; BonDurant *et al.*, 1982; Lindner *et al.*, 1983; Lindner and Ellis, 1985; Leibo and Winniger, 1986). Sreenan *et al.* (1970) reported that cattle embryos could be stored at 10° C for 24 h or longer. Trounson *et al.* (1976a,b) showed a clear relationship between embryo quality and developmental stage and its ability to survive chilling and freezing. They reported that early blastocysts and fully developed blastocysts were apparently resistant to cooling damages whereas the pre-blastocyst stages were not resistant. This finding was supported by Looney *et al.* (1989) who showed that five-day old pre-compacted bovine embryos could not withstand cooling to 4° C. When considering embryo viability and disease transmission, embryo chilling is preferred to embryo freezing for short-term handling.

For the long-term preservation of oocytes and embryos, cryopreservation is the method of choice, as the usual length of cryopreservation time does not affect viability after thawing (Hruska, 1991). Two factors are most important in the cryopreservation of

bovine oocytes and embryos: 1) the rate of cooling and thawing, and 2) the concentration and type of the protective solute or cryoprotectant (Grudzinskas and Yovich, 1995). When cooling cells to subzero temperatures in a physiological medium, ice first forms in the extra-cellular solution and, because of this, the dissolved solutes become more concentrated, as water is removed in the form of ice. The cells respond to equalize the chemical potential of water by losing intracellular water to the extra-cellular solution thereby maintaining osmotic equilibrium. As the temperature falls, the cell contents become increasingly super-cooled and the cell water suddenly freezes within the cells itself (Grudzinskas and Yovich, 1995). Intracellular ice formation damages cell membrane and organelles and causes cell death (Parks and Ruffing, 1992). Seeding or induction of ice formation in the medium overcomes this problem of super-cooling and the deleterious effect of thermal changes following the release of the latent heat fusion (Gordon, 1994; Grudzinskas and Yovich, 1995). If the cells are cooled slow enough, the cells will lose progressively more water across their membrane and this will reduce the intracellular ice formation and cell damage, therefore, cooling rate is an important determinant of the success of cryopreservation (Parks and Ruffing, 1992). Slow freezing and vitrification are commonly used cryopreservation techniques for preservation of embryos and oocytes based on cooling rate. In the slow freezing technique, cells are gradually cooled to sub-zero temperatures in a cryoprotectant media to allow sufficient time for the efflux of intracellular water before it freezes inside the cells. In the commonly adopted slow freezing procedure, the cells are abruptly cooled from room temperature to 0 °C. The cells are then cooled to –6 °C or –7 °C at a rate of 1 °C /min, seeded at –6 °C or –7 °C for 10 minutes, cooled to –35 °C at a rate of 0.3 °C/min and

finally cooled to –196 °C by plunging into liquid nitrogen (Suzuki *et al.,* 1993a,b). After Whittingham *et al.* (1972) and Wilmut *et al.* (1972) first reported the successful cryopreservation of mouse embryos, several reports have been published on cryopreservation of embryos and oocytes from different species using slow freezing technique. Some success has been achieved in the cryopreservation of mouse and human oocytes (Trounson *et al.,* 1986; Sirard *et al.,* 1988). Vitrification is a new cryopreservation technique, in which highly viscous solutions are transformed into a stable, amorphous glass-like structure by rapid cooling, bypassing ice crystal formation while maintaining the properties of a liquid in a solidified form (Rall, 1992). Rall and Fahy (1985) first successfully used this technique to cryopreserve mouse embryos. In this technique, as solutions are changed to glass-like structure and intracellular ice formation is minimal. The major disadvantages in this technique are the use of high concentrations of cryoprotectants and cryoprotectant removal by serial dilutions after thawing. Vitrification can successfully be used to cryopreserve mouse (Nakagata *et al.,* 1989), pig (Arav *et al.,* 1990) and cattle (Massip *et al.,* 1986; Saha *et al.,* 1996, Vajta *et al.,* 1995, 1998) embryos and oocytes. Although, sufficient success was obtained in the cryopreservation of embryos using slow freezing technique, recent findings suggested that vitrification is superior to slow freezing, as manipulated in vitro and in vivo embryos showed similar developmental potential of fresh embryos (Vajta *et al.,* 1998).

Cryoprotectants are broadly divided into intracellular and extracellular solutions. Most cryoprotectants, whether intracellular or extracellular, have similar cryoprotective action, in which they safely remove most of the intracellular water before it freezes inside the cells (Seidel, 1989). They also reversibly depolymerize microfilaments and

microtubules to prevent irreparable destruction to cytoskeletal components (Dobrinsky, 1996). Intracellular cryoprotectants are low molecular weight compounds which are readily penetrable to cells and protect them by preventing potentially deleterious exposure of cells to elevated high concentrations of electrolytes by reducing the quantity of ice formed intracellularly during cryopreservation (Parks and Ruffing, 1992; Grudzinskas and Yovich, 1995; Dobrinsky, 1996). Glycerol, dimethylsulfoxide (DMSO), 1,2-propanediol (PROH) and ethylene glycol (EG) are the most commonly used intracellular cryoprotectants for cryopreservation of embryos (Tachikawa *et al.,* 1993; Takagi *et al.,* 1993; Saha *et al.,* 1996; Vajta *et al.,* 1996, 1998a, b; Ohboshi *et al.,* 1997; Pugh *et al.,* 1998) and oocytes (Lim *et al.,* 1992; Otoi *et al.,* 1992; Fuku *et al.,* 1992; Arav *et al.,* 1993; Schellander *et al.,* 1994; Suzuki *et al.,* 1996; Im *et al.,* 1997: Vajta *et al.,* 1998a; Lim *et al.,* 1999). The permeability of cryoprotectant into oocytes and embryos varies with the species and stage of development (Dobrinsky, 1996). Mogas *et al.* (1997) reported that ethylene glycol tetra-acetic acid (EGTA), a calcium chelator, altered the integrity of junctional complexes between oocytes and its surrounding cells, and allowed faster movement of cryoprotectant. They also obtained high embryo development when exposing bovine oocytes to 1mM EGTA for 5 min before exposing them to cryoprotectant for cryopreservation by slow freezing. Glycerol was first used in the bovine embryo cryopreservation experiments and has higher permeability than DMSO to bovine embryos (Wilmut and Rowson, 1973). Because of the high permeability, glycerol has greater cryoprotective effect on bovine embryos than DMSO (Wilmut and Rowson, 1973). Ethylene glycol has higher permeability than glycerol, DMSO or 1,2-propanediol to bovine embryos and because of this permeability difference, use of ethylene glycol in

bovine embryo cryopreservation gives higher survival rates (Suzuki *et al.,* 1993a,b). One of the main disadvantages to the traditional method of embryo freezing is stepwise removal of cryoprotectant after thawing (Suzuki *et al.,* 1990, 1993a,b). This can be overcome by using ethylene glycol in cryopreservation of bovine embryos with high success rates after one-step thawing (Voelkel and Hu, 1992a,b; Suzuki *et al.,* 1993a,b). This is applicable to both in vivo and in vitro produced bovine embryos (Suzuki *et al.,* 1993a,b; Ooe *et al.,* 1993). Extracellular cryoprotectants are large molecules such as sugars, proteins etc. and little is known about their action. It has been shown that antifreeze glycopeptides isolated from arctic fish could be successfully used as a component of vitrification solutions to cryopreserve bovine and ovine embryos (Arav *et al.,* 1994) as well as porcine (Rubinsky *et al.,* 1991) and mouse (Paynter *et al.,* 1997) oocytes. Paynter *et al.* (1997) also reported that incubation of oocytes in a cryoprotectant with 1mg/ml AFGP at low temperature before cooling for cryopreservation of mouse oocytes gives high embryo development. Saha *et al.* (1994, 1996) reported that carbohydrates could be used as osmotic buffers to maintain osmotic equilibrium between embryonic cells and the external environment in which they were suspended. Saha *et al.* (1996) also reported that biological macromolecules, such as polyvinylpyrrolidone (PVP), were often added to cryoprotective solutions either for their presumptive cryoprotective effect or as a surfactant. Based on these findings, EG is most preferred cryoprotectant for slow freezing of bovine embryos and oocytes, and a mixture of various cryoprotectants with EG is used for vitrification of bovine embryos and oocytes.

The concentration of the cryoprotectant affects the viability of bovine oocytes and embryos by causing osmotic and cytotoxic damages (Parks and Ruffing, 1992; Arav *et*

al., 1993; Fuku *et al.,* 1995; Saunders and Parks, 1999). Ultrastructural changes in the organelles are higher in bovine oocytes at the germinal vesicle stage than that of the 24 h in vitro matured oocytes incubated in DAP213 (2.0M Dimethylsulfoxide (D), 1.0M Acetamide (A) & 3.0M 1,2 propanediol (P)) for 1.5 min (Fuku *et al.,* 1995).

Cooling embryos and oocytes to subzero temperatures can be extremely disruptive to their cellular organization (Parks and Ruffing, 1992; Dobrinsky, 1996). Intracellular ice formation damages various cell organelles which in turn alters basic cell functions. The cytoskeleton, is a network of microfilaments and microtubules that gives structural integrity and is important for intracellular movements, organelle transport and chromosome segregation of cells (Dobrinsky, 1996). Cryoprotectants and cooling methods induces irreversible disruption of microfilaments and microtubules (Overström *et al.,* 1993). Cooling oocytes to freezing temperatures alters the dynamics of microtubules and this may be the reason for low fertilization rate of cryopreserved oocytes (Aman *et al.,* 1994; Albertini *et al.,* 1995). Dobrinsky *et al.* (1996) reported that high embryo survival rates could be obtained when embryos were incubated in 7.5 μg/ml cytochalasin-b to depolymerize microfilaments before exposing them to vitrification solution to prevent irreparable damages to microfilaments. Dilated mitochondria, multiple ruptures in the plasma membrane and accumulation of numerous small vesicles at the cortical region were observed after cryopreservation of bovine oocytes using glycerol (Schmidt *et al.,* 1995). Cooling to subzero temperatures alters the nature of zona pellucida indirectly by causing the premature release of cortical granules, which in turn reduce the fertility of oocytes (Fuku *et al.,* 1992; Hamano *et al.,* 1992; Parks and Ruffing, 1992). Cooling also ruptures the zona pellucida and increases the incidence of polyspermy (Fuku

et al., 1995). The oocyte meiotic spindle is damaged by intracellular ice crystal formation due to low cryprotective effect of cryoprotectant solution or improper cooling rate (Saunders and Parks, 1999). In human (Sathananthan *et al.,* 1988) and animal (Bos-Mikich *et al.,* 1995; Bouquet *et al.,* 1993; Glenister *et al.,* 1987; Richardson *et al.,* 1992; Saunders and Parks, 1999) oocytes, cooling to subzero temperatures causes meiotic spindle disorganization or disruption and chromosomal aberrations, which in turn results in low viability or abnormal embryo development. Although cooling to subzero temperatures causes abnormal embryo development, reports on the production of normal offspring following in vitro fertilization of cryopreserved oocytes are available (Lim et al., 1991; 1992; Otoi et al., 1992; Suzuki et al., 1996). This indicates that the abnormality caused by cooling oocytes to subzero temperatures can be reduced or avoided by improving cryopreservation techniques using different cryoprotectants and cooling methods.

CHAPTER 3 - IN VITRO EMBRYO PRODUCTION USING OVARIES FROM CULLED COWS

3.1 - ABSTRACT

The present study was designed to investigate the oocyte recovery and in vitro embryo production rates in ovaries removed from culled cows at metestrus with or without FSH priming at standing estrus. Animals were ovariectomized 2 d after induced estrus (T_1, n=7), 2 d after a single dose FSH treatment given on the day of induced estrus (T_2, n=7) or randomly irrespective of stage of estrous cycle (T_3, n=7). Oocytes were harvested by needle aspiration, counted, selected and cultured in a maturation media at 38.5°C and 5% CO_2 in a conventional incubator. After 24 h, matured oocytes were incubated with 100μL drops of frozen - thawed semen diluted with BO media to 5 x10^6 sperm/mL at 38.5°C and 5% CO_2 for 16-18 h. Eggs were then cultured in culture media at 38.5°C and 5% CO_2. Cleavage and blastocyst formation rates were recorded after 72 h, and on 7th, 8th and 9th day of culture, respectively. Ovary removal at metestrus did not increase oocyte recovery per ovary, whereas FSH priming at standing estrus significantly increased ($p<0.05$) oocyte recovery rate (T_1=14.6±2.2, T_2=25.4±3.4, T_3=11.1±2.6). Although, cleavage rate (T_1=46.8%, T_2=58.7%, T_3=36.9%) and blastocyst formation rate (T_1=35.4%, T_2=41.0%, T_3=35.4%) were not significantly different among treatments, higher number of blastocysts were obtained from FSH primed animals (T_1=3.1±2.7, T_2=8.3±2.8, T_3=3.3±3.4). The present study indicates that FSH treatment increases oocyte

yield and blastocyst production in cull cows. On average, 6-8 blastocysts can be obtained from culled cows, primed with FSH, using an in vitro embryo production procedure.

3.2 - INTRODUCTION

It is estimated that about 25 to 30 percent of dairy cows are culled every year and end up in slaughterhouses in North America (Bascom and Young, 1998). Culled cows of high genetic merit are potential embryo donors for the embryo transfer industry. Dairy cows produce only five to six calves during their lifetime under the traditional breeding systems. Quite a few reproductive techniques have been developed to increase the number of calves from these animals. Among these, in vivo embryo production technique, which involves superovulation and artificial insemination followed by embryo flushing and transfer, is practically feasible at the farm level (Armstrong, 1993; Calder and Rajamahendran, 1992). But this technique involves expensive hormonal treatments and the number of transferable embryos obtained per flush is highly variable due to variation in response to superovulatory hormone treatment (Adams, 1994; Armstrong, 1993; Foote *et al.,* 1988; Hasler *et al.,* 1983; Monniaux *et al.,* 1983; Moor *et al.,* 1984).

The past decade witnessed considerable advancement in in-vitro maturation, fertilization and culture of oocytes, which resulted in the large-scale production of bovine embryos (Boediono *et al.,* 1994; Brackett and Oliphant, 1975; Krisher and Bovister, 1998; Nicholas, 1996; Schmidt *et al.,* 1996; Torben Greve *et al.,* 1993). Superstimulation followed by ultrasound guided aspiration of follicles is a rapidly developing technique for collection of oocytes from live animals of high potential genetic merit for in vitro embryo

production (Bordignon *et al.,* 1997; Fry *et al.,* 1998; Looney *et al.,* 1994; Santl *et al.,* 1998). This technique requires expensive equipment and trained personal. In the absence of these facilities, the other alternative is to remove the ovaries and produce embryos from culled cows in vitro before the animals are sent to slaughter. Pedigree and clinical history of culled animals are very important criteria to use them effectively for in vitro embryo production to avoid transmission of genetic defects and diseases.

Ultrasonographic studies have shown that there are two or three waves of follicular growth during the bovine estrous cycle (Sirois and Fortune, 1988; Savio *et al.,* 1988; Ginther *et al.,* 1989; Fortune, 1994). In each follicular wave, a dominant follicle is selected for further growth and the subordinate follicles undergo atresia (Sirois and Fortune, 1988; Ginther *et al.,* 1989, 1996; Sunderland *et al.,* 1994; Fortune, 1994; Bodensteiner *et al.,* 1996). The first follicular wave emerges immediately after estrus and is associated with a small surge of FSH (Adams *et al,* 1992; Sunderland *et al.,* 1994; Bodensteiner *et al.,* 1996; Ginther *et al.,* 1996). The dominant follicle of the first follicular wave shows rapid growth 2 to 3 d post estrus and exerts inhibitory effects on subordinate follicles through both direct and indirect mechanisms (Ginther *et al.,* 1996). Based on ultrasonographic studies of the ovaries, the best time for the removal of ovaries from culled cows for maximum oocyte recovery and developmental competence is during the metestrus period (2 d after standing estrus). FSH priming during the endogenous FSH surge is also shown to increase the number of viable oocytes and follicles by reducing the atresia or increasing the number of follicles recruited (Calder and Rajamahendran, 1992).

In this experiment, we attempted to maximize embryo production from culled Holstein dairy cows by using in-vitro techniques for maturation, fertilization and culture

of eggs. We also hypothesized that removal of ovaries during the early part of the first follicular wave would yield many viable oocytes and embryos, and FSH priming at the time of endogenous FSH surge could potentiate this yield.

3.3 - MATERIALS AND METHODS

3.3.1 - Animals and Treatments

Cycling, lactating Holstein cows, which were ear marked for culling, from two research farms belonging to The University of British Columbia were used in this experiment. A total of 21 animals were randomly selected and assigned to three treatments and the experimental design was as follows:

Treatment 1 (T_1) - Seven cows were treated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (25 mg Lutalyse (The Upjohn Company – Animal Health Division, Orangeville, Ontario, Canada), i.m.) to induce estrus and ovaries were removed 2 d after standing estrus.

Treatment 2 (T_2) - Seven cows were treated with $PGF_{2\alpha}$ (25 mg Lutalyse, i.m.) to induce estrus and were primed with FSH (40 mg Folltropin (Vetrepharm Canada Inc., London, Ontario, Canada), i.m.) at standing estrus and ovaries were removed 2 d after standing estrus.

Treatment 3 (T_3) – Seven cows were not treated and ovaries were removed irrespective of the day of estrous cycle.

3.3.2 - Collection of Ovaries and Oocytes

The ovaries were removed through the vagina using an ecraseur (Colpotomy technique) under epidural anesthesia (Drost *et al.,* 1992) and brought to the laboratory in a Thermos flask containing sterile physiological saline at 30 - 35° C within 4 h of collection. Oocytes from small follicles were aspirated into follicle aspiration medium (Appendix 1.), which was Dulbecco's phosphate buffered saline (DPBS) (GIBCO BRL, Life Technologies), 0.3 % bovine serum albumin (BSA) (Sigma Pharmaceuticals) and 50 μg/ml gentamicin (Sigma Pharmaceuticals), using an 18G needle and a 10 ml syringe. The total number of oocytes obtained from each animal was recorded. The good quality cumulus oocyte complexes (COC), the oocytes surrounded by more than three layers of cumulus cells and evenly granulated cytoplasm (Plate 3.1.(A)), were selected for maturation and the number of oocytes selected from each animal was recorded (Loose *et al.,* 1989; Lonergan *et al.,* 1994).

3.3.3 - In vitro Maturation, Fertilization and Culture

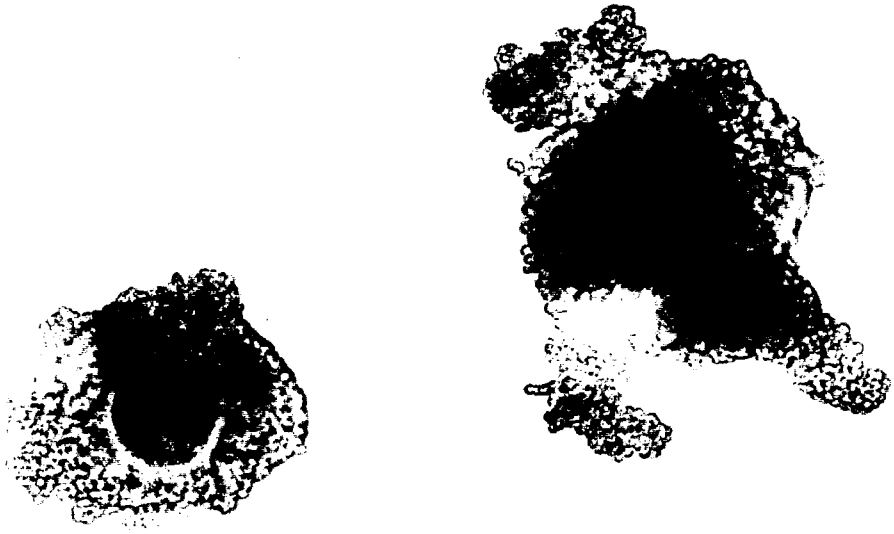
Selected oocytes were cultured in maturation medium (Appendix 1), which was TCM199 (Sigma Pharmaceuticals) supplemented with 0.01 mg/ml FSH (Folltropin V, Vetripharm, London, Ontario, Canada), 5% superovulated cow serum (SCS) (Boediono *et al.,* 1994) and 50 μg/ml gentamicin (Sigma Pharmaceuticals), in a 5% CO_2 incubator (Forma Scientific) at 38.5 °C for 24 h (Plate 3.1.(B)). Frozen semen (Westgen) from a single bull was thawed at 37 °C, washed twice by centrifugation at 500g for 5 min, then diluted to 5 x 10^6 sperm/ml in Brackett and Oliphant medium (BO) (Appendix 2.)

(Brackett and Oliphant, 1975), which was supplemented with 2.5 mM caffiene sodium benzoate and 20 μg/ml heparin. Semen droplets (100 μl) were prepared under mineral oil and pre-incubated for 1 h. After 24 h of complete maturation, 20-30 oocytes were placed in each of these semen droplets and incubated in a 5% CO_2 incubator at 38.5 °C for 16-18 h. The eggs were then cultured on a cumulus cell layer in culture media (Appendix 2) which was prepared by mixing TCM-199, 5% SCS, 5 μg/ml insulin and 50 μg/ml gentamicin (Boediono *et al.,* 1994), in four well culture dishes in 5% CO_2 incubator at 38.5 °C. The culture media was changed every 72 h. The cleavage and blastocyst formation rates were recorded 72 h after insemination, and on the 7th, 8th and 9th day of embryo culture, respectively (Plate 3.2.(A),(B)).

3.3.4 - Statistical Analysis

Treatment groups were compared for average oocyte yield per ovary, percent oocyte selected for maturation, percent eggs cleaved 72 h after insemination and percentage embryos developed to blastocyst stage up to 9 d of culture after fertilization. The data were statistically analyzed using ANOVA after arcsine transformation and significant treatment means were separated using the Least Square Difference method.

A)



B)

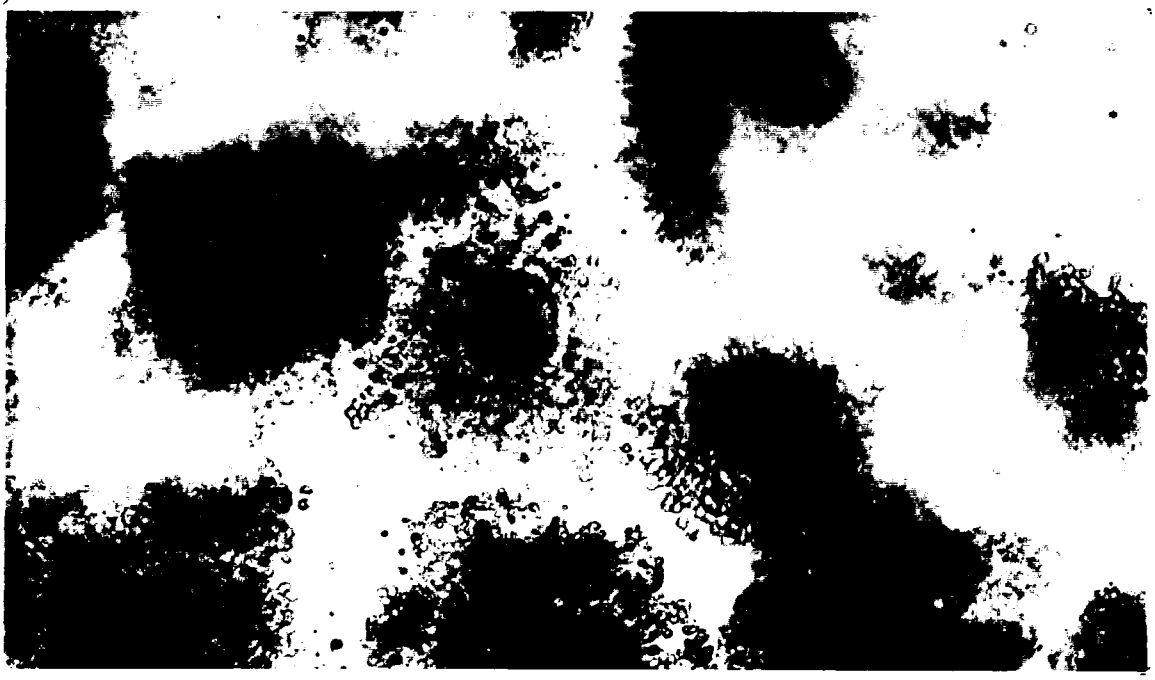


Plate 3.1. Bovine immature oocytes immediately after aspiration and mature oocytes with expanded cumulus cells approximately 24 h after maturation.
A) Good quality immature oocytes with more than three layers of cumulus investment (100x).
B) Mature oocytes with expanded cumulus cells (100x)

A)

B)

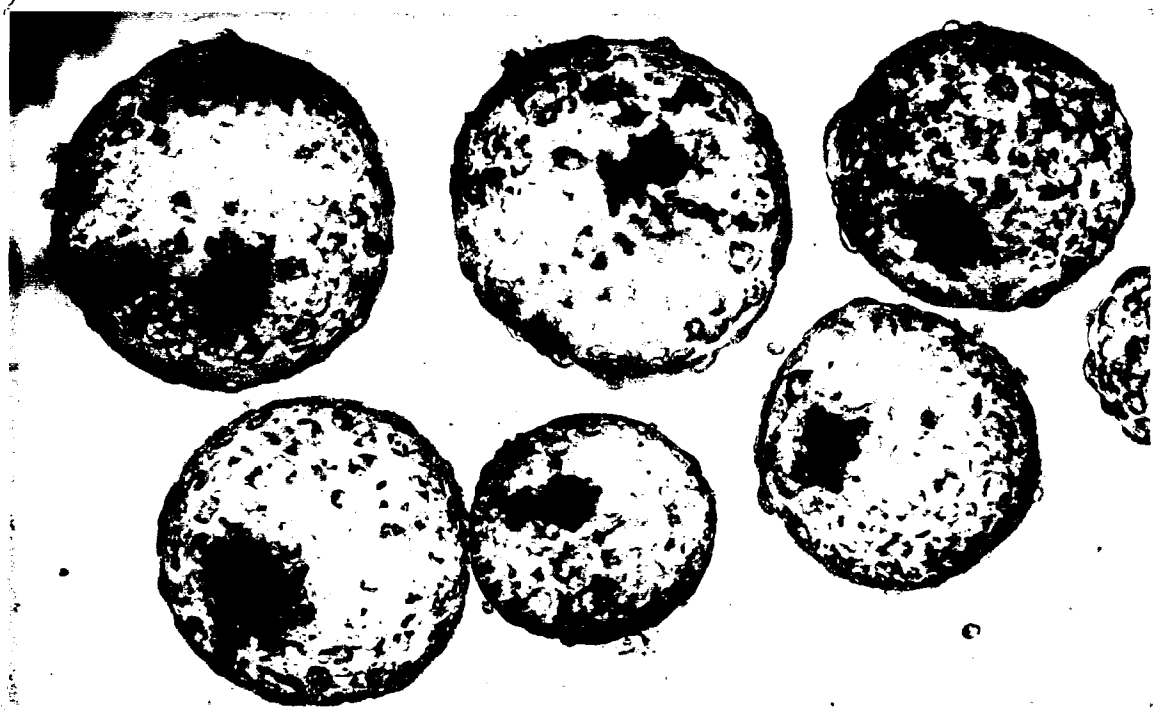
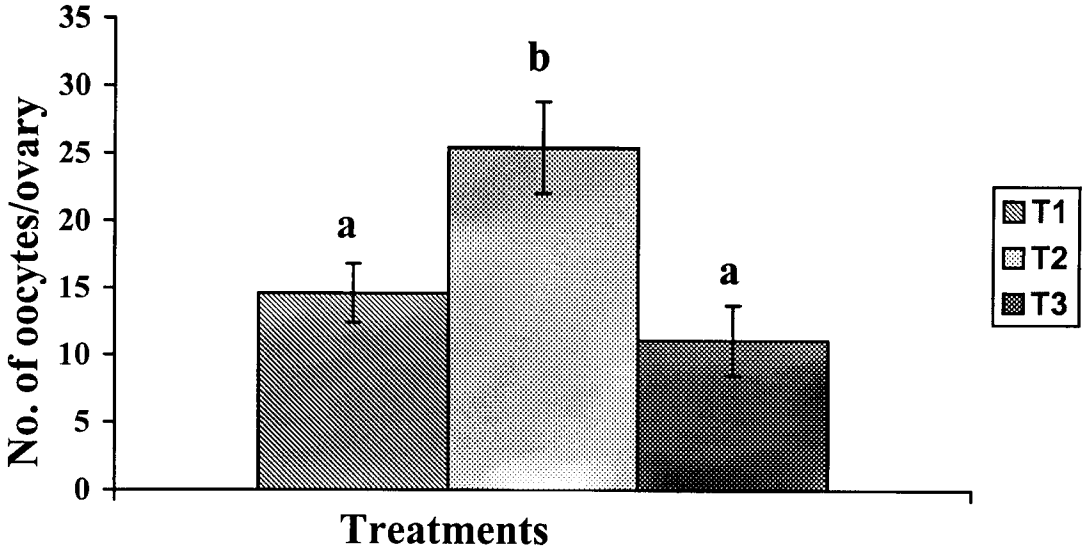


Plate 3.2. Bovine blastocysts and hatched blastocysts 7-9 d after fertilization
A) Blastocysts approximately 7-9 d after fertilization (200x)
B) Hatched blastocysts approximately 9 d after fertilization (200x)

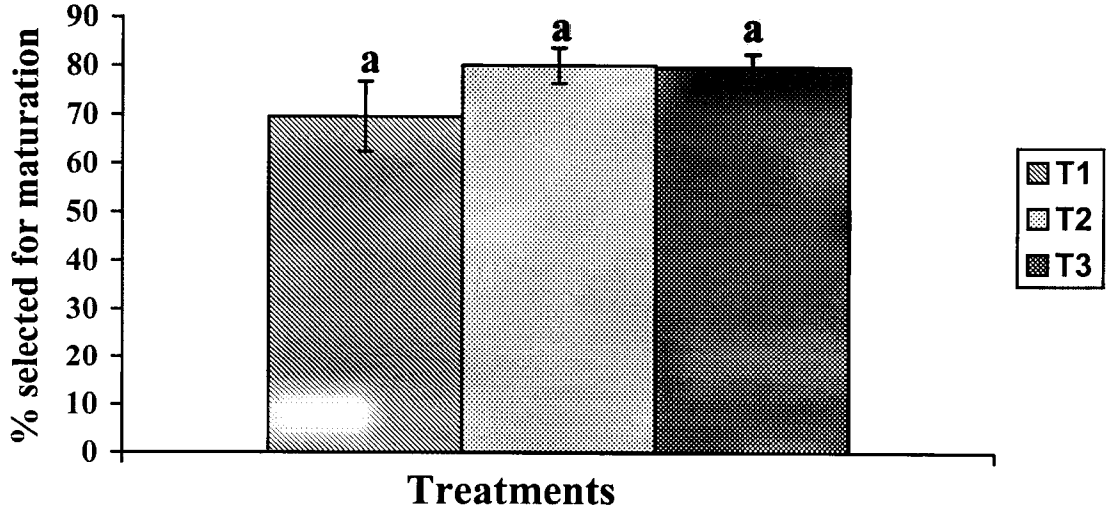
3.4 - RESULTS

Results of ovary removal at the early part of first follicular wave with and without FSH priming on oocyte recovery, oocyte selection, cleavage rate and in vitro embryo production rate are shown in Figures 3.1 and 3.3. FSH priming at the day of standing estrus significantly increased ($P<0.05$) the oocyte recovery rate (Figure 3.1.A.) (T_1=14.6±2.2, T_2=25.4±3.4, T_3=11.1±2.6). However, ovary removal at metestrus with or without FSH treatment did not increase the percentage of oocytes selected for maturation ($P>0.05$) (T_1=69.5±7.1, T_2=79.9±3.6, T_3=79.5±2.7) (Figure 3.1.B.), percentage embryos cleaved 72 h after insemination (T_1=46.8±5.0, T_2=58.7±5.6, T_3=36.9±7.8) (Figure 3.2.A.), or percent blastocyst formed up to 9 d after insemination (T_1=35.4±10.5, T_2=41.0±4.1, T_3=35.4±10.3) (Figure 3.2.B.).

A)

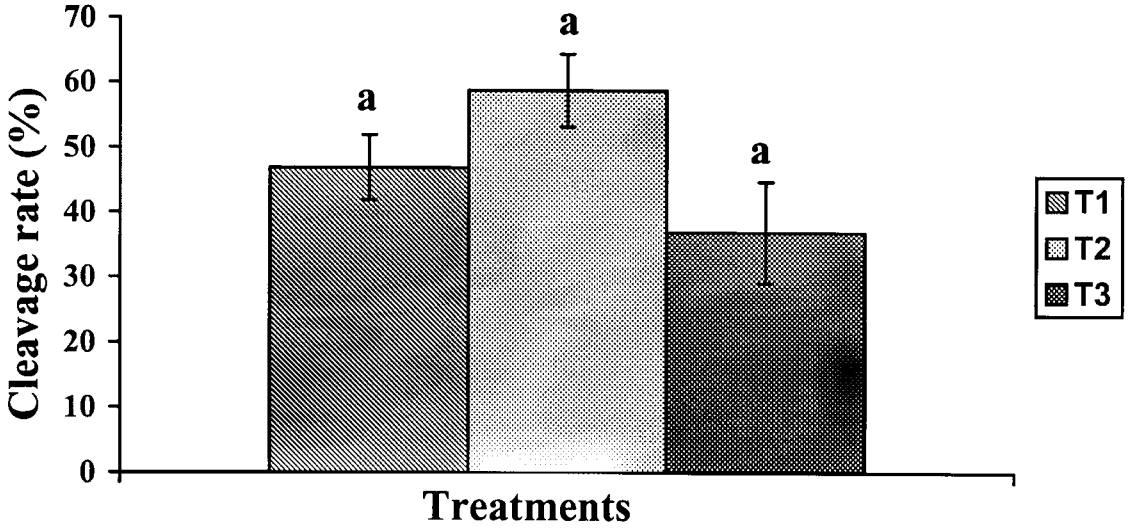


B)

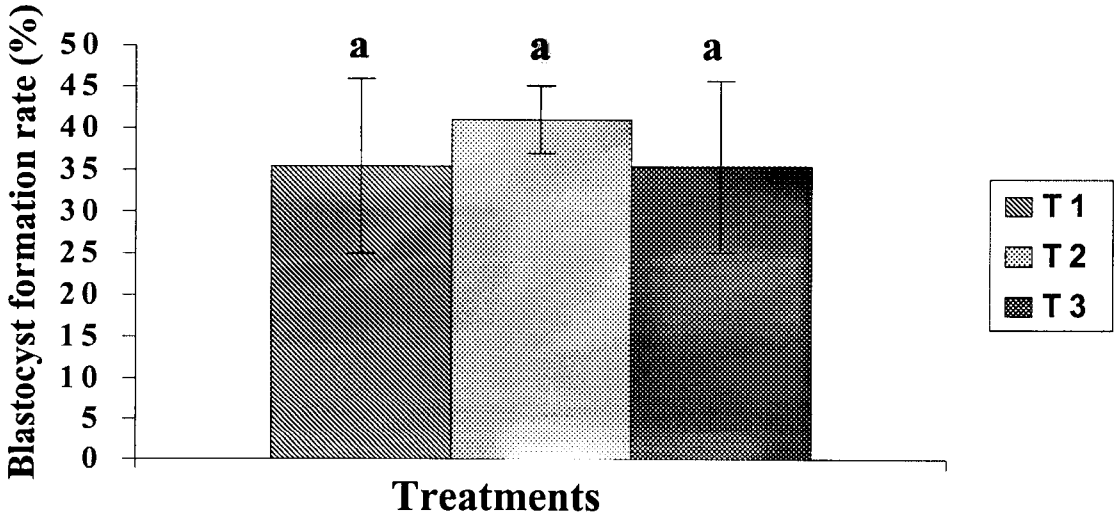


Figures 3.1. - Effect of stage of estrous cycle and FSH priming on the day of standing estrus on oocyte yield (A), oocyte selection for maturation (B). T_1 – Culled Holstein cows treated with $PGF_{2\alpha}$ and ovaries removed 2 d after estrus (n=7). T_2 – Culled Holstein cows treated with $PGF_{2\alpha}$, and FSH on the day of estrus and overiectomised 2 d after estrus (n=7). T_3 – Culled Holstein cows overiectomised without $PGF_{2\alpha}$ or FSH treatment irrespective of the day of estrous cycle (n=7). Bars with different letters differ significantly ($P<0.05$).

A)



B)



Figures 3.2. - Effect of stage of estrous cycle and FSH priming on the day of standing estrus on cleavage rate (A) and blastocyst formation rate (B). T_1 – Culled Holstein cows treated with $PGF_{2\alpha}$ and ovaries removed 2 d after estrus (n=7). T_2 – Culled Holstein cows treated with $PGF_{2\alpha}$, and FSH on the day of estrus and overiectomised 2 d after estrus (n=7). T_3 – Culled Holstein cows overiectomised receiving no treatment of $PGF_{2\alpha}$ or FSH and irrespective of the day of estrous cycle (n=7). Bars with same letters do not differ significantly ($P<0.05$).

DISCUSSION

In this experiment, ovaries were removed during metestrus with and without FSH priming on the day of standing estrus to maximize in vitro embryo production from culled cows.

The oocyte recovery rate using needle aspiration was similar to results obtained from other studies (Carolan *et al.,* 1994). In the FSH primed cows, the number of oocytes was almost double that of the untreated cows (T_3). This observation indicated that FSH priming increased the number of follicles recruited during the first follicular wave. Therefore, this technique could be used to increase the production of embryos from culled cows. Collection of oocytes using needle aspiration only yields 10-15 oocytes per ovary with 45 - 50 percent acceptable quality (Katska and Smorak, 1984; Carolan *et al.,* 1994), whereas the ovary-slicing method yields 40-60 oocytes per ovary with 70 - 75 percent acceptable quality (Xu *et al.,* 1992a, b; Stringfellow *et al.,* 1993). Ovary slicing method is time consuming and difficult to apply for large-scale collection of oocytes. If one or two cull cows are used at a time for in vitro embryo production, the time factor can be avoided and the oocyte recovery rate can be increased using the ovary-slicing method. The in vitro embryo production rate can also be increased by combining FSH priming on the day of standing estrus with the ovary slicing technique for oocyte collection.

Studies have shown that semen from different bulls and different ejaculates will effect in vitro fertilization and in vitro embryo development (Eyestone and First, 1989a; Shi et al., 1990; Hillery et al., 1990; Saacke et al., 1994; Kurtu et al., 1996; Larocca et al., 1996). In this study, I used semen from a single ejaculate of one bull and this might be the

reason for lower cleavage and blastocyst formation rates than those reported in other studies (Suzuki *et al.,* 1996). Therefore, if semen from a highly fertile bull was used for this operation we might have obtained a higher number of viable embryos from culled cows.

At present, semen sexing is a rapidly growing technique for the pre-selection of offspring for desired sex (Johnson *et al.,* 1994). By using sexed semen, embryos of desired sex can be produced from these valuable cows using an in vitro embryo production technique.

There was no significant difference in the percentage of oocytes selected for maturation. This indicated that, although FSH priming increased the number of follicles recruited during first follicular wave, the percent viability of the oocytes was not changed.

Although, there is no statistically significant difference in cleavage rate among treatment groups, there is approximately a 10 % increase in cleavage rate of T_1 compared to that of T_3. FSH priming also increased the cleavage rate by about 20 and 10 % that of T_3 and T_1, respectively. This indicates that there is a potential for the increment of viability of oocytes used in my treatments, but, because of low sample number and high variability among animals, these values were not statistically different. Future studies with a larger sample number could reveal the actual effect of collection of oocytes during the early part of first follicular wave and FSH priming at the day of standing estrus on viability and further development of oocytes after in vitro fertilization.

Some studies suggested that age of animal influence the number of follicles recruited at a time and the viability of oocytes (Lerner *et al.,* 1986). My studies showed that age of animal does not influence the number of follicles recruited at a time as most of

the animals used in this experiment were old but the viability of these follicles was lower than expected. This can be attributed to the effects of the ovulatory dominant follicle on newly recruited follicles because the presence of a dominant follicle during FSH priming reduced the response of other follicles and increased atresia of newly recruited follicles (Wolfsdorf *et al.,* 1997). Future studies with FSH priming after ablation of the dominant follicle by ultrasound guided needle aspiration will verify the effect of the ovulatory dominant follicle on newly recruited follicles (Stubbings and Walton, 1995).

In conclusion, FSH priming on the day of standing estrus increases follicular recruitment and thereby increases in vitro embryo production. This study offers a protocol for the production of in vitro embryos from good and excellent producing cows after culling for preservation of their genetics and for international market at significantly reduced cost to a greater number of producers.

CHAPTER 4 - MATURATION OF BOVINE OOCYTES IN STRAWS: A METHOD FOR TRANSPORTING OOCYTES

4.1 - ABSTRACT

The objective of this study was to identify a suitable maturation environment that would enable long distance transport of bovine oocytes for successful in-vitro embryo production. Oocytes were aspirated from bovine ovaries obtained at a local abattoir. Selected oocytes were assigned to three treatment groups for in vitro maturation. In treatment 1 (T_1), oocytes (n=527) were matured in 0.5 ml straws containing maturation media (MM) at 38.5 °C and 5% CO_2 in a conventional incubator. In treatment 2 (T_2), oocytes (n=527) were matured in 0.5 ml straws containing MM at room temperature in a Styrofoam container. In treatment 3 (T_3), oocytes (n=527) were matured in MM in culture dishes at 38.5 °C and 5% CO_2 in a conventional incubator. After 24 h, 20 to 25 matured oocytes from all treatments were incubated with 100 µl drops of frozen - thawed semen diluted with Brackett and Oliphant (BO) medium to 5 x10^6 sperm/ml in a conventional incubator for 16-18 h. Eggs were then cultured under optimum culture conditions. Cleavage and blastocyst formation rates were recorded for each treatment group, after 72 h and on the 7th, 8th and 9th day of culture after insemination, respectively. Nuclear staining was carried out before maturation, 24 h after maturation, 12 h after fertilization with HOECHST stain and maturation and fertilization rates were recorded (n=540). Analysis of variance procedure and least square difference were used to analyze data and separate treatment means, respectively. Maturation rate (T_1=83.3%, T_2=36.7%, T_3=86.7%), fertilization rate (T_1=78.3%, T_2=41.7%, T_3=85.0%), cleavage rate (T_1=53.7%,

T_2=29.4%, T_3=56.3%) and blastocyst formation rate (T_1=37.8%, T_2=6.1%, T_3=55.5%) were significantly reduced ($P<0.05$) in T_2. Maturation, fertilization and cleavage rates were not significantly different between T_1 and T_3 (83.3% Vs 86.7%, 78.3% Vs 85.0%, 53.7% Vs 56.3%) whereas blastocyst formation rate of T_1 was significantly lower ($p<0.05$) than that of T_3 (39.3% Vs 51.9%). From these results, I conclude that 5% CO_2 and 38.5 °C temperature are necessary for optimum blastocyst production.

4.2 - INTRODUCTION

For the large-scale laboratory production of bovine embryos, in-vitro techniques for oocyte maturation, fertilization and embryo culture have been established during the past decades (Brackett *et al.,* 1982; Torben Greve *et al.,* 1993; Looney *et al.,* 1994; Nicholas, 1996; Schmidt *et al.,* 1996; Krisher and Bovister, 1998). Proper collection, preservation and maturation of oocytes are important for the success in in-vitro embryo production. Although successful methods have been established to preserve semen in the past, a successful method for the preservation of oocytes has not been established yet. Methods used for freezing and thawing affect the post thaw survival and developmental competence of the oocytes (Lim *et al.,* 1992; Otoi *et al.,* 1992; Parks and Nancy, 1992; Dobrinsky, 1996). A few authors have reported that frozen oocytes can be used to establish pregnancy but the success rate was low in these studies (Lim *et al.,* 1991, 1992, 1999; Otoi *et al.,* 1992; Suzuki *et al.,* 1996; Vajta *et al.,* 1998).

At present, a high demand exists for developmentally competent oocytes for advanced reproductive research (Campbell *et al.,* 1996; Heyman and Renard, 1996;

Moens *et al.,* 1996; Wilmut *et al.,* 1997). Optimum time duration for storage of bovine ovaries after slaughter is 0-8 h and the oocytes should be aspirated within this time to obtain a maximum number of viable oocytes but this is not always practical (Yang, N.S., *et al.,* 1990). The overall objective of this study is to develop a new maturation system for the successful transportation of oocytes without affecting the viability of oocytes until arrival at the destination.

Most in vitro embryo production techniques showed that the ideal condition for maturation of oocytes is culturing oocytes in a bicarbonate/CO_2 buffered environment (Staigmiller and Moor, 1984; Moore and Gondolfi, 1987; Crozet, 1991). However, it has been shown that oocytes can also be matured in a non- 5% CO_2 environment and produce viable embryos (Westhusin *et al.,* 1992; Byrd *et al.,* 1997). Therefore, in this experiment, we hypothesized that maturing oocytes in straws without 5% CO_2 at 38.5 °C would not effect the viability of oocytes and embryos.

4.3 - MATERIALS AND METHODS

4.3.1 - Collection of Ovaries and Oocytes

Ovaries were collected from a local slaughterhouse and brought to the laboratory in sterile physiological saline, containing streptomycin (0.2 mg/ml) and penicillin (100 IU/ml), in a Thermos flask at 30-35 °C within 4 h (Saha *et al.,* 1996). The small follicles were aspirated into follicle aspiration media (Appendix 1.), containing phosphate buffered saline supplemented with 0.3% bovine serum albumin (BSA) and 50 μg/ml

gentamicin, by using an 18 gauge needle and a 10 ml syringe (Carolan *et al.,* 1994, Saha *et al.,* 1996). Good quality cumulus oocyte complexes (COC), the oocytes surrounded by more than three layers of cumulus cells, and uniformly regular cytoplasm, were selected (Loose *et al.,* 1989; Lonergan *et al.,* 1994) and randomly assigned for each treatment.

4.3.2 - In Vitro Maturation

Treatment 1 (T_1) - Oocytes (30-40) were kept in each of 0.5 ml straws with maturation media (MM) (Appendix 1.), containing TCM199 supplemented with 5% SCS, FSH and 50 μg/ml gentamicin, at 38.5 °C and 5% CO_2 in a conventional incubator for 24 h after heat-sealing both ends.

Treatment 2 (T_2) - Oocytes (30-40) were kept in each of 0.5 ml straws with MM at room temperature (25 °C) in a Styrofoam box for 24 h after heat-sealing both ends.

Treatment 3 (T_3) - Oocytes were kept in a small culture dish with MM at 38.5 °C and 5% CO_2 in a conventional incubator for 24 h.

4.3.3 - In Vitro Fertilization and Culture

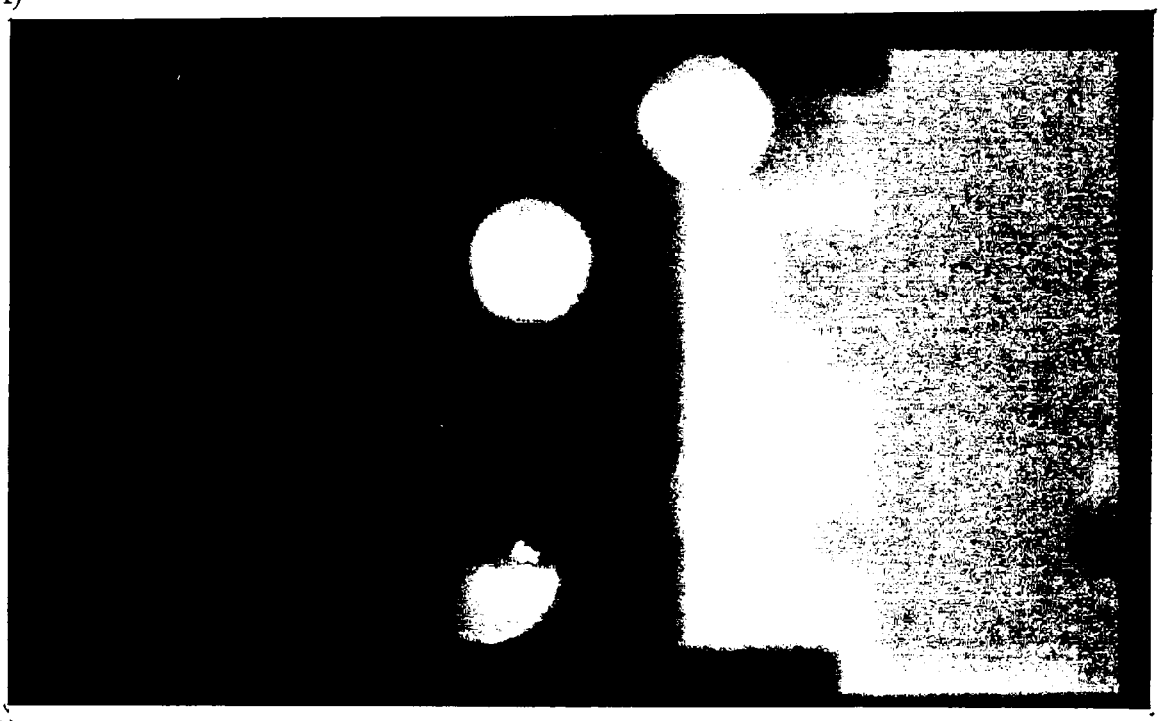
After 24 h maturation, oocytes were incubated in 100 μl semen droplets which were diluted with BO medium (Appendix 2) (Brackett and Oliphant, 1975; Saha *et al.,* 1996) to 5 x 10^6 sperm/ml, at 38.5°C and 5% CO_2 for 16-18 h. The eggs were incubated on a cumulus cell layer in culture media (Appendix 2) containing TCM 199 supplemented with 5% SCS (Suzuki and Shimohira, 1985; Boediono *et al.,* 1994), 0.5

μg/ml insulin and 50 μg/ml gentamicin, in a four well culture dish at 38.5°C and 5% CO_2. The culture media was changed after 72 h of culture (Boediono *et al.,* 1994). The cleavage and blastocyst formation rates were recorded 72 h after insemination, and on 7th, 8th and 9th day of embryo culture, respectively.

4.3.4 - Nuclear Staining

To stain the nucleus of the oocytes, the differential fluorochrome staining method used by Saha *et al.* (1996) to stain embryos was used with slight modification. The cumulus cells were removed from oocytes by pipetting them in and out through a narrow pipette. The zona pellucida was removed by incubating oocytes in 0.25 % pronase diluted in TCM 199 for 5 – 10 min. The zona free oocytes were then stained by incubating them in 20 μg/ml bisbenzimide solution (HOECHST stain) for 30 min. and observed under fluoroscent microscope at 100x magnification (Saha *et al.,* 1996). Any oocytes showing clear nuclei or germinal vesicles were considered as immature oocytes (Plate 4.1.(A)). Oocytes showing a polar body and metaphase spindle were considered as mature oocytes (Plate 4.1.(B)). The zygotes showing two pronuclei and polar bodies were considered monospermic (Plate 4.2.(A)). The zygotes with more than two pronuclei were considered polyspermic (Plate 4.2.(B)) (Suzuki *et al.,* 1996).

A)



B)

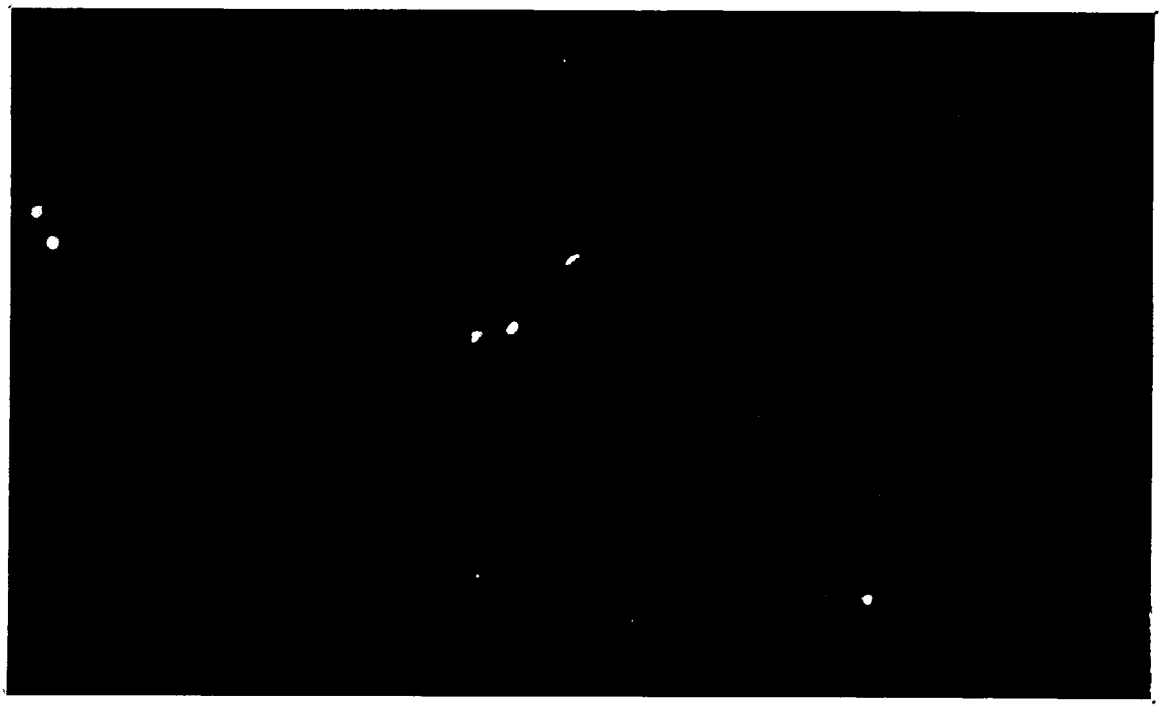
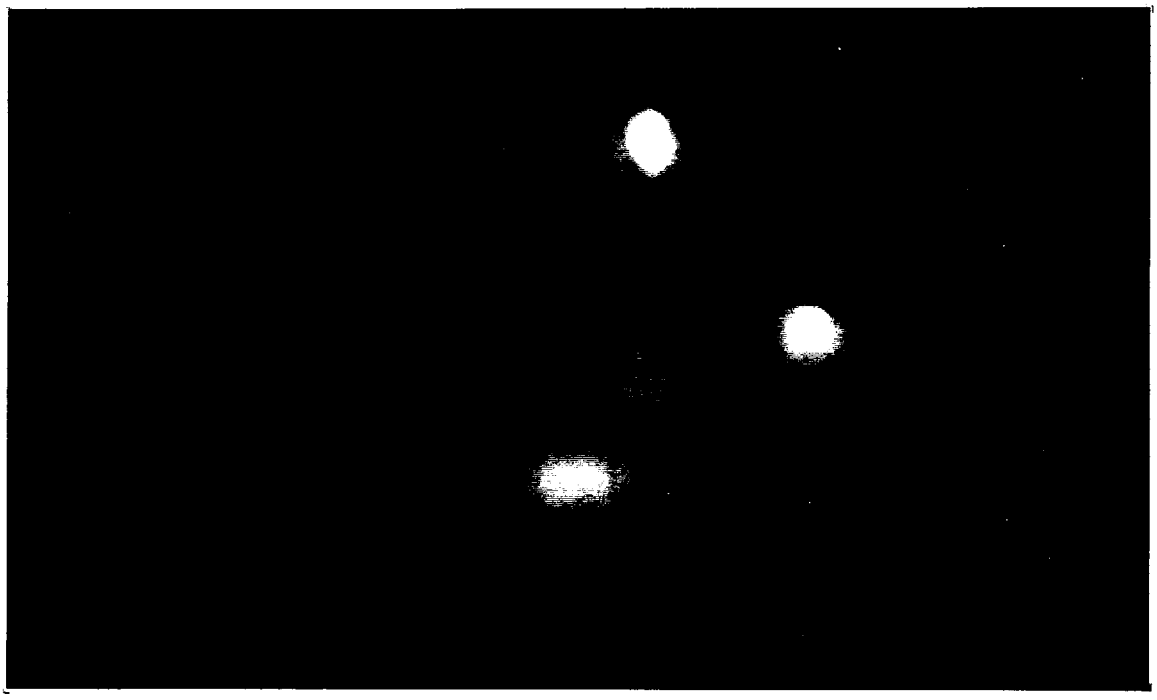


Plate 4.1. Bovine mature and immature oocytes after staining with HOECHST stain
A) Immature oocytes with germinal vesicle(100x)
B) Mature oocytes with metaphase plate and first polar body (100x)

A)



B)

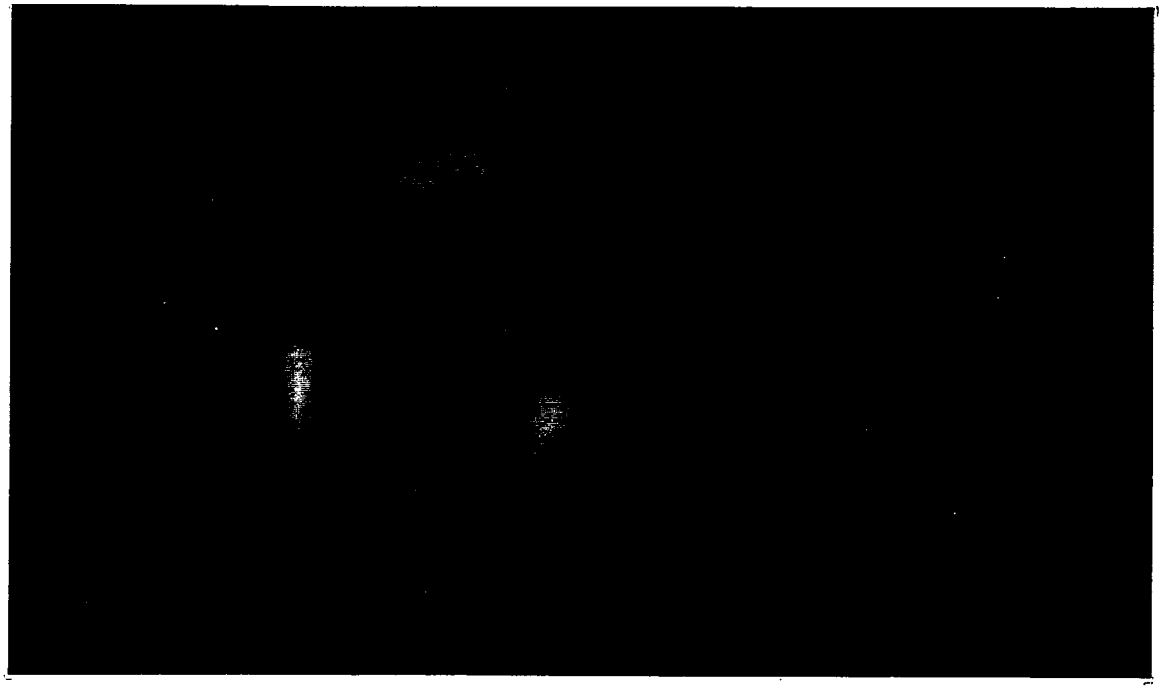


Plate 4.2. Bovine oocytes stained with HOECHST stain approximately 14-16 h after fertilization

A) Monospermic fertilization with male and female pronuclei (400x)

B) Polyspermic fertilization with more than two pronuclei (400x)

4.3.5 - Experimental Design

Three trials were conducted with a total of 540 oocytes for nuclear staining to determine maturation and fertilization rates. Eight trials were conducted with a total of 1581 oocytes to determine cleavage and blastocyst formation rates. Treatments were compared for maturation, fertilization, cleavage, and blastocyst formation rates. Treatment groups were organized in a randomized complete block design and statistically compared by ANOVA procedure. Between treatment comparisons were done using the least square difference method.

4.4 - RESULTS

Results of this experiment are shown in Table 1 and Figure 1. The oocytes kept at room temperature showed a significant ($p < 0.05$) reduction in maturation (36.7%), fertilization (41.7%), cleavage (29.4%) and blastocyst formation rates (6.1%). The cleavage rate was not different between oocytes kept in straws or culture dishes at 38.5°C and 5% CO_2 (53.7%, 56.5%, respectively). Oocytes kept in straws showed a significant reduction ($p<0.05$) in blastocyst formation rate (38.7%, 55.5%., respectively).

Table 4.1. - Effect of different maturation methods on maturation and fertilization rates. Maturation and fertilization rates were determined after nuclear staining with bisbenzimide (HOECHST stain).

Treatments	No. of oocytes/trial	Maturation rate (%) Mean±SEM	Fertilization rate (%) Monospermy Mean±SEM	Polyspermy Mean±SEM	Unfertilized Mean±SEM
T_1	60	83.3±3.3[a]	78.3±1.7[a]	13.3±1.7[a]	8.3±1.7[a]
T_2	60	36.7±3.3[b]	41.7±4.4[b]	6.7±1.7[b]	51.7±4.4[b]
T_3	60	86.7±3.3[a]	85.0±2.9[a]	11.7±1.7[a]	3.3±3.3[a]

T_1 – Oocytes were matured in 0.5 ml straws at 38.5° C without 5% CO_2.
T_2 – Oocytes were matured in 0.5 ml straws at 25° C without 5% CO_2.
T_3 – Oocytes were matured in culture dishes 38.5° C with 5% CO_2.
[a, b] Values within columns with different superscripts are significantly different ($p<0.05$)

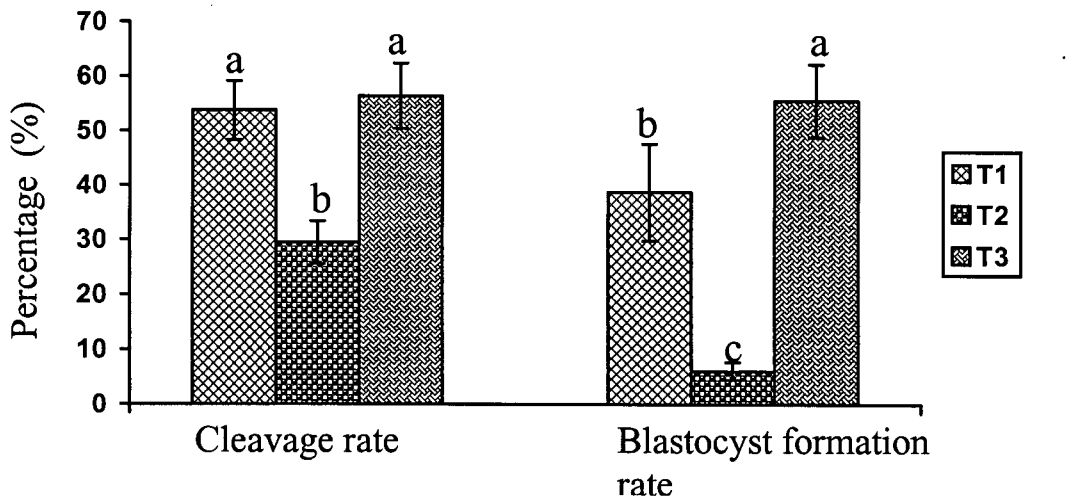


Figure 4.1. - Effect of different maturation methods on cleavage rate and blastocyst formation rate. T_1 – Oocytes were matured in 0.5 ml straws at 38.5° C without 5% CO_2. T_2 – Oocytes were matured in 0.5 ml straws at 25° C without 5% CO_2. T_3 – Oocytes were matured in culture dishes 38.5° C with 5% CO_2. Cleavage and blastocyst formation rates were determined after in vitro fertilization and culture. a,b,c - Bars with different letters are significantly different ($p<0.05$).

4.5 - DISCUSSION

I conducted this experiment with straws because it was easy to transport embryos in straws than in culture plates.

The embryonic genome is activated when embryos reach the 8 to 16 cell stage (Telford *et al.,* 1990). This maternal-zygotic transition or embryonic genome activation is initiated by various inter-contacts between embryos and the surrounding media with the involvement of growth factors and their receptors (Watson *et al.,* 1992b). It has been shown that, in the final stages of maturation, the genome of the maturing oocyte is very active in the production of messenger RNAs and proteins for early embryonic development. It has also been shown that some of the growth factors, receptors and their messenger RNAs are produced during the final stages of maturation (Watson *et al.,* 1992b). These findings indicate that proper oocyte maturation is important for successful development of embryos after fertilization. In my study, I found that maturing oocytes without 5% CO_2 had no effect on subsequent embryo development up to 72 h of culture and had a significant effect on embryo development from 72 h to 9 d of culture. Similar observations on embryo development up to 72 h after fertilization were reported in one study but in contrast to my findings, subsequent embryo development was not affected by maturing oocytes without 5% CO_2 (Byrd *et al.,* 1997). The low developmental potential in my studies indicate that there may be a problem in the activation of the early embryonic genome and this may be due to low production of growth factors, receptors and their messenger RNAs during maturation.

Culture temperature has a significant effect on maturation of bovine oocytes and their subsequent development (Lenz *et al.,* 1983). A range of temperatures between 35 °C and 39 °C are accepted as the optimum temperatures for maturation of bovine oocytes (Lenz *et al.,* 1983; Katska and Smarog 1985; Morstin and Katska 1986). However, some studies show that the optimum temperature for in vitro maturation of bovine oocytes is 38-39 °C (Wang *et al.,* 1991). These findings are supported by our findings in which the oocytes matured at room temperature showed a significant reduction in maturation, fertilization, cleavage and blastocyst formation rates. This indicates that temperature is much more important than 5% CO_2.

Reduced developmental potential of T_2 oocytes after 72 h might be attributed to the accumulation of toxic materials in the straws as 20-30 oocytes were loaded per straw and straws were sealed at both ends. More studies have to be carried out with low numbers of oocytes per straw to verify whether embryo development up to the blastocyst stage is actually affected by maturing oocytes without 5% CO_2 and low temperature.

In my study, the cleavage rate of control oocytes was lower than that reported by other authors. This may be due to the high variability among trials. Although our results showed a statistically significant reduction in blastocyst formation rate, a considerable number of oocytes matured without 5% CO_2 developed to the blastocyst stage. On account of the high cost of embryo production, cryopreservation and transport, this technique can be used to transport oocytes as ovaries can be obtained from slaughterhouse at a low cost.

The results of this experiment indicate that an optimum temperature and 5% CO_2 are necessary during maturation for overall embryo development. Contradictory reports

discrediting the need for 5% CO_2 during maturation for further embryo development are available in the literature. Therefore, further studies should be conducted to confirm our results.

CHAPTER 5 – CRYOPRESERVATION OF BOVINE OOCYTES

5.1 - ABSTRACT

The present trend in gamete research is to find out a suitable method for preservation of female germ plasm for various advanced reproductive research as well as preservation of valuable genetic material to establish ova banks. In this experiment, I attempted to develop a suitable cryopreservation method for long-term preservation of bovine oocytes. In the first part of the experiment, selected immature oocytes were either slow frozen (T_1, n=100), 24 h matured and slow frozen (T_2, n=100) or 24 h matured (T_3, n=100) in vitro. For slow freezing, 10 to 15 oocytes were loaded into straws and slow frozen after 10 min incubation in 1.8 M ethylene glycol (EG) solution supplemented with 0.3% BSA, 0.05 M trehalose (T) and 5% PVP by using a programmable freezer. In the second part of the experiment, selected immature oocytes were either vitrified (T_1, n=90), 24 h matured and vitrified (T_2, n=90), exposed to vitrification solution and 24 h matured (T_3, n=90), or 24 h matured (T_4, n=90) in vitro. For vitrification, 10 to 15 oocytes were loaded into straws and vitrified by direct plunging into liquid nitrogen after 10 min and 30 sec incubation in low concentration and high concentration vitrification solutions, respectively. Low concentration vitrification solution was a mixture of DPBS, 0.25 M dimethylsulfoxide (DMSO), 0.375 M dimethylacetamide (DMA), 0.875 M EG, 0.3% BSA and 0.05 M T. High concentration vitrification solution was a mixture of DPBS, 1 M DMSO, 1.5 M DMA, 3.5 M EG, 0.3% BSA, 0.3 M T and 20% PVP. Slow frozen and vitrified oocytes were thawed and immature oocytes were matured for 24 h. The oocytes were then fertilized and cultured using standard in vitro embryo production protocols developed in our laboratory. The cleavage rate and blastocyst formation rates were

recorded 72 h, and 7, 8 and 9 days of culture after insemination, respectively. The analysis of variance procedure was used to analyze the data and least square difference was used to separate treatment means. None of the slow frozen mature oocytes and vitrified immature and mature oocytes cleaved 72 h after insemination. Although some of the slow frozen immature and immature oocytes cleaved 72 h after insemination, the cleavage rate was significantly lower ($P<0.05$) than that of control oocytes (T_1 - 9.7±0.3, T_2 - 5.6±5.6, T_3 - 50±15). None of the slow frozen and vitrified immature and mature oocytes developed to blastocyst stage. Although some of the vitrification solution exposed immature oocytes cleaved 72 h after insemination, the cleavage rate (T_3 – 63.3±5.1, T_4 – 81.1±4.9) and blastocyst formation rate (T_3 – 11.1±1.1, T_4 – 15.6±1.1) were significantly lower ($P<0.05$) than that of control oocytes. The cryopreservation procedure decreased the developmental competency of bovine oocytes. This effect might be due to cryoprotectant, cooling or both.

5.2 - INTRODUCTION

Since oocytes are large germ cells produced in very low numbers, cryopreservation of oocytes is more difficult than that of spermatozoa or embryos (Grudzinskas and Yovich, 1995). However, advanced reproductive technologies, such as in vitro embryo production, embryo cloning, transgenic animal production, require a plentiful supply of viable, developmentally competent oocytes. Although a surplus amount of oocytes can be obtained from slaughterhouses, their usage is limited because of the lack of preservation methods. Finding out a suitable long-term preservation method for bovine oocytes will help to overcome this problem as well as to preserve genetically valuable female germ plasm to create ova banks.

Since the successful cryopreservation of mouse oocytes by Whittingham (1977), scientists have tried to find a suitable cryopreservation method for bovine oocytes (Heyman *et al.,* 1986; Lim *et al.,* 1991, 1992, 1999; Fuku *et al.,* 1992; Otoi *et al.,* 1992, 1995; Schellander *et al.,* 1994; Martino *et al.,* 1996; Suzuki *et al.,* 1996; Mogas *et al.,* 1997; Im *et al.,* 1997; Otoi *et al.,* 1997; Vajta *et al.,* 1998; Hochi *et al.,* 1998). Although successful production of live offspring from slow frozen bovine oocytes by in vitro fertilization and culture was reported a decade ago (Lim *et al.,* 1991; 1992; Fuku *et al.,* 1992; Otoi *et al.,* 1992), the success rate in this technology has still been very low compared to fresh oocytes. Since the report on cryopreservation of bovine oocytes using glycerol and gradual cooling by Lim *et al.* (1991,1992), various cryoprotectants have been employed in the slow freezing protocol for the cryopreservation of bovine oocytes. Otoi *et al.* (1992) and Fuku *et al.* (1992) used 1,2 propanediol for the preservation of bovine oocytes with little success in development to the blastocyst stage after in vitro fertilization and culture. Comparison of different cooling rates for the cryopresevation of bovine oocytes after exposure to 1.6 M 1,2 propanediol revealed that cooling rates below 1 °C/min did not affect post thaw developmental competence of oocytes after in vitro fertilization and culture (Otoi *et al.,* 1994). Also, comparison of cryoprotective effects of DMSO, 1,2 propanediol and glycerol for the slow freezing of mature bovine oocytes revealed that 1,2 propanediol was superior to other two cryoprotectants for the development of bovine oocytes after thawing and in vitro fertilization (Lim *et al.,* 1999). After thawing, proper cryoprotectant removal reduces osmotic shock and improves the viability of oocytes (Hamano *et al.,* 1992). Sucrose and other carbohydrates have commonly been used for removal of cryoprotectant from oocytes. Cryoprotectant

removal, after cryopreservation using glycerol, propyleneglycol and dimethylsulfoxide, and thawing of bovine oocytes, with sucrose, lactose and trehalose showed that no difference in development of oocytes (Schellander *et al.,* 1994). Addition of biological macromolecules such as carbohydrates, PVP to cryoprotectant media improves the post thaw survival of bovine oocytes (Saha *et al.,* 1996). Use of 1.8 M EG, 0.3% BSA, 0.05 M T and 5% PVP for the cryopreservation of bovine germinal vesicle stage oocytes by slow freezing gave the maximum embryo development after thawing and in vitro fertilization (Suzuki *et al.,* 1996). In the first part of the experiment, we examined the repeatability of the EG based slow freezing protocol in our lab for the preservation of mature and immature bovine oocytes.

The other method of cryopreserving bovine oocytes is by vitrification, which was first successfully used by Rall and Fahy (1985) for the preservation of mouse embryos. In the vitrification procedure, higher concentrations of cryoprotectant solutions are changed to a glass-like structure by rapid cooling. In this procedure, ice crystal formation is minimal. Although vitrification reduces damage caused by intracellular ice crystal formation, higher concentration of cryoprotectant causes toxic damage to oocytes. To overcome this problem, a combination of low concentrations of different cryoprotectants can be used in the vitrification protocol. A combination of 2 M DMSO, 1 M acetamide and 3 M propylene glycol (DAP213) was successfully used to vitrify mature bovine oocytes, which developed to blatocysts and gave pregnancies after in vitro fertilization and culture (Hamano *et al.,* 1992). In their studies, the success rate was very low after vitrification of bovine oocytes using solution DAP213. This may be due to toxic effects of high concentrations of DMSO (Fuku *et al.,* 1995). In DAP213, acetamide is

incorporated to reduce the toxicity of high concentrations of DMSO but the glass forming tendency of acetamide is very low as it has only one methyl group (Fahy *et al.,* 1985). Addition of a methyl group to the carbonyl carbon of formamide or N-methyl fromamide enhanced basicity of the carbonyl oxygen and acidity of the N-M proton available for the H_2O interaction. Another basis for the methylation effect may be interference with self-association of the cryoprotectant (Fahy *et al.,* 1985). Addition of a methyl group to the nitrogen may tend to reduce this association, producing more opportunities for interaction with water, and similar arguments are applicable to polyols. These effects are correlated with enhanced glass forming tendency (Nielson *et al.,* 1982; Spencer *et al.,* 1980 & 1981). Since DMA has a similar ability as acetamide in reducing toxicity of DMSO and has more methyl groups than acetamide (Fahy *et al.,* 1985), combining DMSO with DMA gives better cryoprotection than combining it with acetamide. DMA was also reported as an excellent cryoprotectant for cryopreservation of poultry semen, since its usage in the preservation of semen from most domestic poultry species gave consistent high fertility rates (Kurbatov *et al.,* 1986; Surai *et al.,* 1996). In these studies, the optimum concentration used for the cryopreservation of semen to get higher fertlity was 8-10% DMA (Kurbatov *et al.,* 1986; Surai *et al.,* 1996). As ethylene glycol (EG) has a higher penetrating ability than propanediol (Fahy *et al.,* 1985) and propanediol has an effect of parthenogenetic activation (Suzuki *et al.,* 1996), better cryoprotection might be obtained by using ethylene glycol instead of propylene glycol in the DAP213 vitrification mixture. Carbohydrates can be added to cryoprotectant media as osmotic buffers to maintain osmotic equilibrium between embryonic cells and the external environment (Saha *et al.,* 1994, 1996). Biological macromolecules such as PVP, are also often added

to cryoprotective solutions for their presumptive cryoprotective effects or as a surfactant (Saha *et al.,* 1996). Higher survival rates can be obtained by vitrifying bovine embryos using 40% EG, 0.3 M Trehalose (T) and 20% PVP (Saha *et al.,* 1996). In the second part of the experiment, I prepared and examined a new combination of 1 M DMSO, 1.5 M DMA and 3.5 M EG with 0.3 M T and 20% PVP based on these studies and DAP213 mixture for the vitrification of bovine oocytes.

Damages caused by incubation in different cryoprotectant solutions and very low temperature cooling of bovine oocytes for cryopreservation vary with the stage of maturation. Lim *et al.* (1992) showed that oocytes at different nuclear stages had different developmental potentials after cryopreservation by slow freezing. They also showed that mature oocytes have higher developmental potential than oocytes at germinal vesicle stage. Fuku *et al.* (1992) used 2.0 M 1,2 propanediol for slow freezing and DAP213 for vitrification of bovine oocytes and reported that in vitro matured oocytes have a higher developmental potential than germinal vesicle stage oocytes after cryopreservation by slow freezing. They also showed that developmental potential of cryopreserved oocytes by slow freezing was higher than that of the cryopreserved oocytes by vitrification. In this experiment, I used 0 h matured and 24 h matured oocytes to find out the effect of stage of maturation and either slow freezing or vitrification with new formulation vitrification solution on developmental competence of oocytes.

5.3 - MATERIAL AND METHODS

5.3.1 - Collection of Ovaries and Oocytes

The ovaries were collected from a slaughterhouse and brought to the laboratory in a Thermos flask containing sterile physiological saline at 35 °C within 4 h. Oocytes from small follicles were aspirated into follicle aspiration media (Appendix 1) using an18G needle and a 10 ml syringe. Good quality COCs, where the oocytes were surrounded by more than three layers of cumulus cells and had an evenly granulated cytoplasm, were selected (Loose *et al.,* 1989; Lonergan *et al.,* 1994) and randomly assigned to each treatment.

5.3.2 - Slow Freezing

Randomly selected and either 24 h matured or immature (germinal vesicle stage) oocytes were placed in freezing media, which was composed of DPBS containing 1.8 M EG, 0.05 M T, 0.3% BSA and 5% PVP at room temperature for 10 min. Then 15 to 20 oocytes were loaded into 0.25 ml plastic straws. The oocytes were cooled directly from room temperature to 0 °C. After 2 min exposure at 0 °C, the oocytes were cooled to –6 °C at a rate of 1 °C/min, seeded at –6 °C for 10 min and again cooled to –30 °C at a rate of 0.3 °C/min using a programmable freezer. Then the straws were directly plunged into liquid nitrogen (Suzuki *et al.,* 1996).

5.3.3 - Vitrification

The oocytes were suspended in a low concentration vitrification solution (LVS), which was a mixture of DPBS, 0.25 M DMSO, 0.375 M DMA, 0.875 M EG, 0.3% BSA and 0.05 M T, at room temperature for 10 min and transferred to high concentration vitrification solution (HVS), which was a mixture of DPBS, 1 M DMSO, 1.5 M DMA, 3.5 M EG, 0.3 M T, 0.3% BSA and 20% PVP and kept at 4 °C. After 30 sec exposure to HVS at 4 °C, 15 oocytes were loaded into 0.25 ml plastic straws and the straws were directly plunged into the liquid nitrogen.

5.3.4 - Thawing

Cryopreserved oocytes were thawed by keeping the straws in air for 5 sec and in water bath at 37 °C for 10 sec. The vitrified oocytes were diluted first in LVS for 10 min at room temperature and washed three times in DPBS containing 0.3% BSA. Then GV oocytes were transferred to MM for complete maturation and 24 h mature oocytes were transferred to fertilization media. Slow frozen oocytes were diluted and washed three times in DPBS containing 0.3% BSA. Then the GV stage oocytes were transferred to MM for complete maturation and 24 h mature oocytes were transferred to fertilization media.

5.3.5 - In Vitro Maturation, Fertilization and Culture

Oocytes were cultured in MM in a 5% CO_2 incubator at 38.5 °C for 24 h. After 24 h complete maturation, 20-30 oocytes were incubated in 100 µl semen droplets, prepared as in the previous experiments, in a 5% CO_2 incubator at 38.5 °C for 16-18 h. Then they were incubated on a cumulus cell layer in culture in four well culture dish in a 5% CO_2 incubator at 38.5 °C. The culture media was changed every 72 h. The cleavage and blastocyst formation rates were recorded 72 h, and on the 7th, 8th and 9th day of embryo culture after insemination, respectively (Suzuki *et al.*, 1996).

5.3.6 - Experimental Design

Experiment 1a

This experiment, repeated slow freezing technique reported by Suzuki *et al.* in 1996, was examined to preserve bovine oocytes without compromising their viability in our laboratory. In this experiment, 300 selected oocytes were randomly assigned to three different treatments in three trials as follows:

Treatment 1(T_1) – Oocytes were frozen before maturation (GV stage), thawed, matured, fertilized and cultured

Treatment 2 (T_2) – Oocytes were frozen 24 h after maturation (metaphase II stage), thawed, fertilized and cultured

Treatment 3(T_3) – Oocytes were matured fertilized and cultured without any treatment

Experiment Ib

In this experiment, a new formulation of vitrification solution for successful vitrification of bovine oocytes was investigated. To reduce the toxicity of vitrification solution, a mixture of cryoprotectant solutions in low concentrations was used vitrification solution. In this experiment, 360 selected oocytes were randomly assigned to four different treatments in three trials as follows:

Treatment 1 (T_1) – Oocytes were vitrified before maturation, thawed, matured, fertilized and cultured.

Treatment 2 (T_2) – Oocytes were vitrified 24 h after maturation, thawed, fertilized and cultured

Treatment 3 (T_3) – Oocytes were only exposed to vitrification solutions as in T_1 and T_2, matured, fertilized and culture

Treatment 4 (T_4) – Oocytes were matured fertilized and cultured without any treatment

The data were analyzed by using analysis of variance procedure and the means were separated using least square difference technique.

5.4 - RESULTS

Results of this experiment are shown in Tables 5.1, and 5.2., and Figures 5.1. and 5.2. None of the slow frozen mature oocytes and vitrified immature and mature oocytes cleaved by 72 h after insemination. Although some of the slow frozen immature oocytes cleaved 72 h after insemination, the cleavage rate was significantly lower ($P<0.05$) than that of control (T_3) oocytes (T_1-9.7±0.3, T_2-5.6±5.6, T_3-50±15). None of the slow frozen and vitrified immature and mature oocytes developed to blastocyst stage. Although some

of the immature oocytes exposed to the vitrification solution cleaved 72 h after insemination, the cleavage rate (T_3-63.3±5.1, T_4-81.1±4.9) and blastocyst formation rate (T_3-11.1±1.1, T_4-15.6±1.1) were significantly lower ($P<0.05$) than that of control (T_4) oocytes. The cryopreservation procedure decreased the developmental competency of bovine oocytes.

Table 5.1. – Effect of stage of maturation and slow freezing on developmental competence of bovine oocytes.

Treatment	Total	# Cleaved (Mean%±SEM)	# of blastocysts (Mean%±SEM)
T_1	100	10 (9.7±0.3) [a]	0 (0±0) [a]
T_2	100	5 (5.6±5.6) [a]	0 (0±0) [a]
T_3	100	53 (50±15) [b]	14 (13.6±2.2) [b]

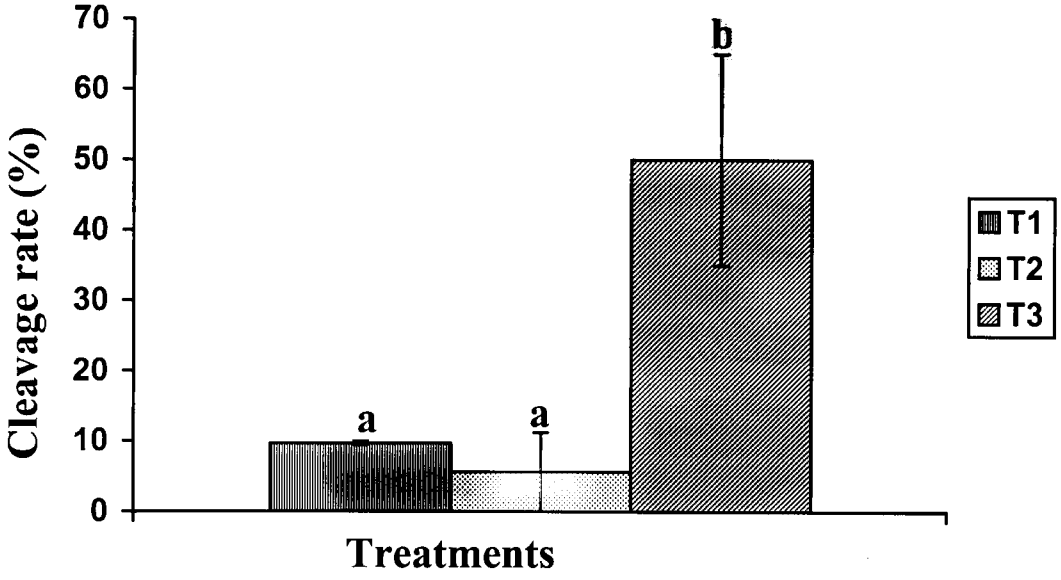
T_1 – Slow frozen immature oocytes matured, fertilized and cultured in vitro after thawing.
T_2 – Slow frozen mature oocytes fertilized and cultured in vitro after thawing.
T_3 – Normally in vitro matured, fertilized and cultured oocytes.
[a,b] -Values within columns with different superscripts differ significantly ($P<0.05$)
SEM – Standard error of treatment means.

Table 5.2. – Effect of stage of maturation and vitrification on developmental competence of bovine oocytes.

Treatment	Total	# Cleaved (Mean%±SEM)	# of blastocysts (Mean%±SEM)
T_1	90	0 (0±0) [a]	0 (0±0)
T_2	90	0 (0±0) [a]	0 (0±0)
T_3	90	57 (63.3±5.1) [b]	10 (11.1±1.1) [a]
T_4	90	73 (81.1±4.9) [c]	14 (15.6±1.1) [b]

T_1 – Vitrified immature oocytes matured, fertilized and cultured in vitro after thawing.
T_2 – Vitrified mature oocytes fertilized and cultured after thawing.
T_3 – Immature oocytes only exposed to vitrification solution and matured, fertilized and cultured in vitro.
T_4 - Normally in vitro matured, fertilized and cultured oocytes.
[a,b] -Values within columns with different superscripts differ significantly ($P<0.05$)
SEM – Standard error of treatment means

A)



B)

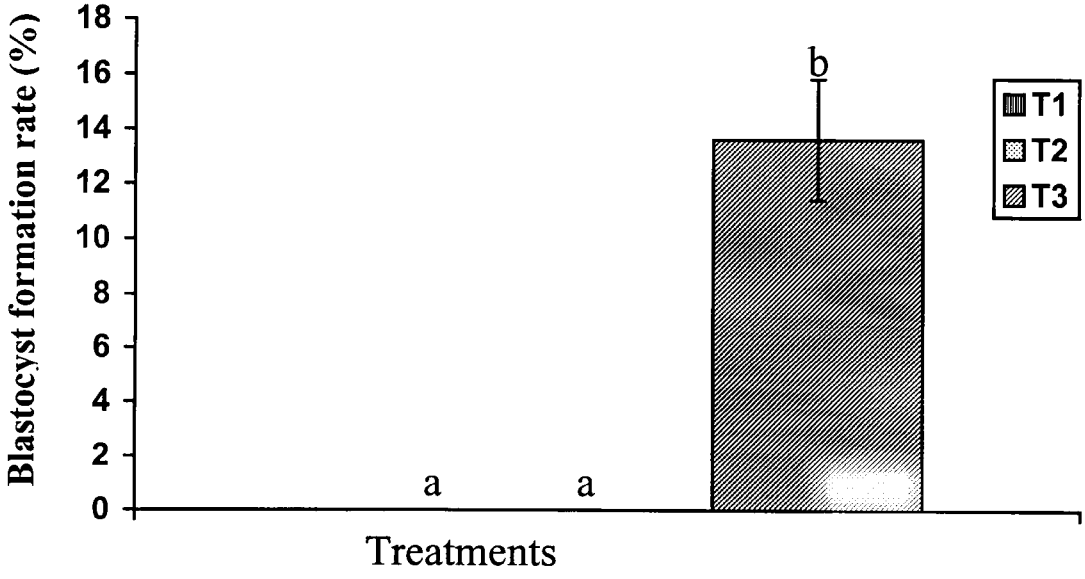
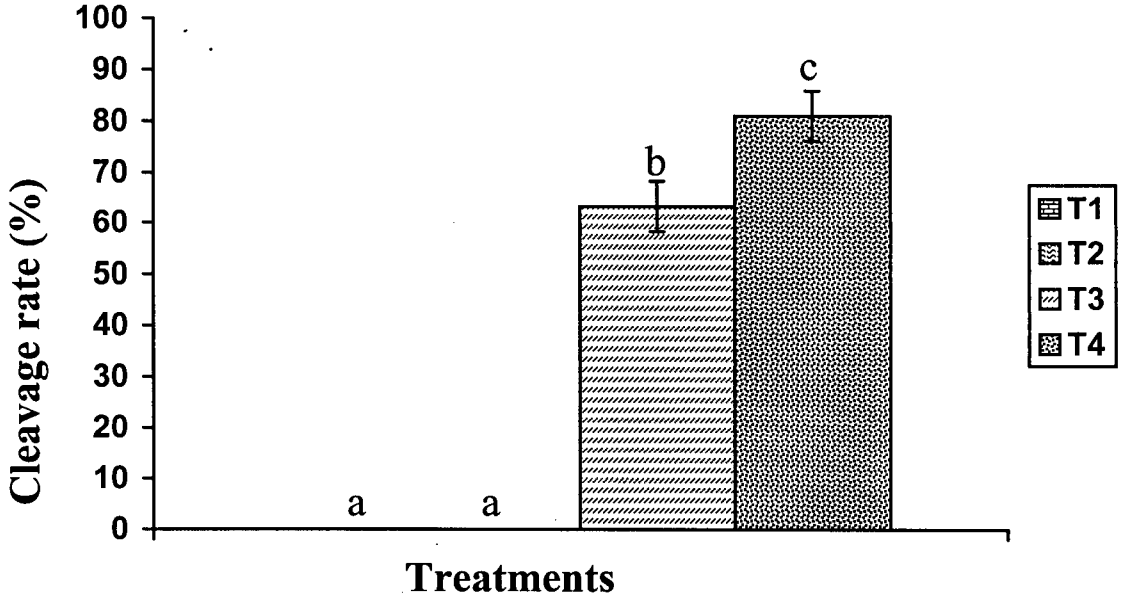


Figure 5.1. – Effect of stage of maturation and slow freezing on cleavage (A) and blastocyst formation (B) rates of bovine oocytes. T_1 – Slow frozen immature oocytes matured, fertilized and cultured in vitro after thawing, T_2 – Slow frozen mature oocytes fertilized and cultured in vitro after thawing and T_3 – Normally in vitro matured, fertilized and cultured oocytes. a,b – Bars with different letters differ significantly ($P<0.05$).

A)



B)

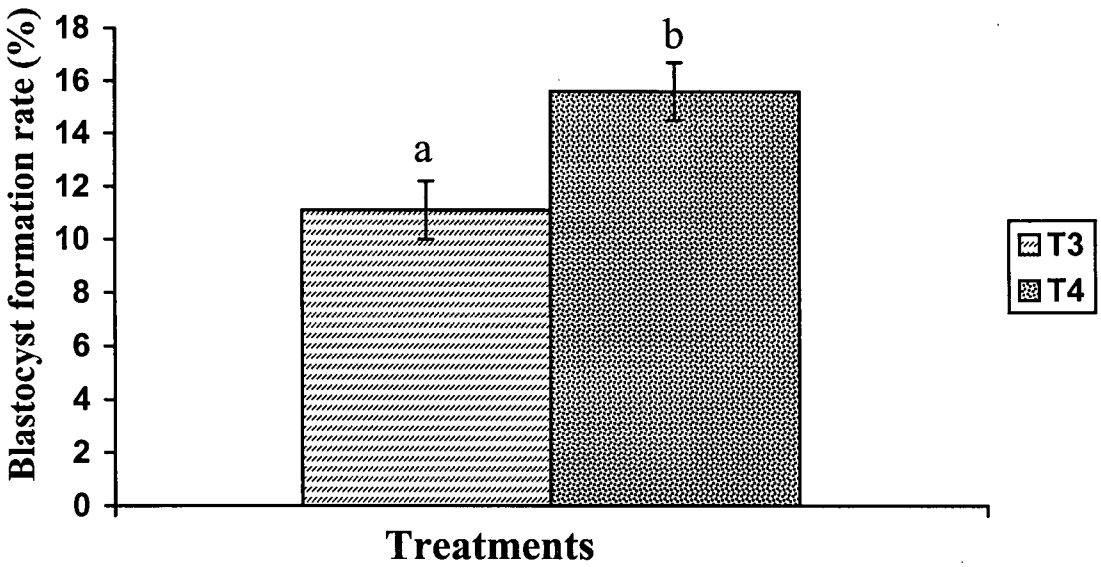


Figure 5.2. – Effect of stage of maturation and vitrification on cleavage (A) and blastocyst formation (B) rates of bovine oocytes. T_1 – Vitrified immature oocytes matured, fertilized and cultured in vitro after thawing, T_2 – Vitrified mature oocytes fertilized and cultured after thawing, T_3 – Immature oocytes only exposed to vitrification solution and matured, fertilized and cultured in vitro, and T_4 - Normally in vitro matured, fertilized and cultured oocytes. a,b -Bars with different letters differ significantly ($P<0.05$).

5.5 – DISCUSSION

Bovine oocytes are very sensitive to cooling and display very little tolerance for specific effects of cryoprotectants, chilling and freezing which leads to low success rate of cryopreservation of bovine oocytes. Intracellular structures such as meiotic spindle, cytoskeletal elements, cortical granules, mitochondria and zona pellucida of the bovine oocytes are altered by freezing and thawing (Parks and Ruffing, 1992). The meiotic spindle of the matured oocyte is very sensitive to cooling (Parks and Ruffing, 1992; Otoi *et al.,* 1997). My results clearly indicate very low developmental competence of frozen thawed oocytes reflecting that the intracellular structures of oocytes were altered because of intracellular ice formation or osmotic stress.

The slow freezing procedure, which I adopted, was similar to the procedure reported by Suzuki *et al.* (1996). In their studies, they demonstrated that about 20 % of the immature oocytes cleaved after slow freezing, thawing, and in vitro maturation, fertilization and culture developed to blastocyst. In my studies, although very few oocytes showed cleavage, none of the slow frozen oocytes developed to blastocyst stage. This indicates very poor repeatability of this technique.

Vitrification is a convenient method of cryopreserving biological tissues, as this technique takes less time and needs less expensive equipment. In my experiment, to reduce the toxicity of the vitrification solution DAP213 in my new formulation, I planned to use DMA and ethylene glycol instead of acetamide and propanediol and to reduce the concentration of DMSO. Since ethylene glycol is less toxic and highly penetrable than

propanediol and DMA has high glass forming tendency and neutralizes the toxicity of DMSO, we expected high survival rate of bovine blastocyst after vitrification.

In the vitrification procedure, higher concentrations of cryoprotectant solutions are used to increase the viscosity. High concentration cryoprotectant solutions cause osmotic and cytotoxic damages to oocytes. Hence, proper removal of the cryoprotectant after thawing is important for a high success rate in vitrification. It has been shown that serial dilution and removal of cryoprotectant with macromolecules, such as sucrose and trehalose, after thawing reduces osmotic damage and gives high survival rates (Fuku *et al.,* 1995). In my vitrification protocol, I used a single step dilution, because I used a combination of low concentration cryoprotectants, I assumed single step dilution was sufficient to remove the cryoprotectants from oocytes. This one step dilution might be the reason for low survival rates in my experiments.

Like cooling, the thawing procedure is also important for the successful preservation of bovine embryos because, if the thawing speed is not sufficient, intracellular ice forms and causes damage to intracellular structures (Otoi *et al.,* 1996; Vajta *et al.,* 1998). It has been also shown that rapid thawing procedures using open pulled straws reduced the damages caused by intracellular ice formation and thus increased the success rate in the cryopreservation of bovine oocytes (Vajta *et al.,* 1998a, b). In my study, I used a thawing protocol, which was commonly used for slow freezing, and this might cause intracellular ice formation and damaged intracellular structures.

Exposure to cryoprotectants was shown to alter structure of the bovine oocytes. Exposure of bovine oocytes to DAP213 for 1.5 min showed premature exocytosis of cortical granules and low fertilization, cleavage and blastocyst formation rates after in

vitro insemination (Fuku *et al.,* 1995). This finding is supported by my studies in which immature bovine oocytes exposed to vitrification solution showed significant reduction in cleavage and blastocyst production rates.

In conclusion, my study indicated that slow freezing and vitrification with new formulation vitrification solution reduce developmental competence of mature and immature bovine oocytes, similarly, and this reduction in viability may be due to high sensitivity of oocytes to cryoprotectants and chilling. Further studies with ultra-rapid thawing, and serial dilution and removal of cryoprotectants are necessary to verify low success rate in the vitrification procedure.

CHAPTER 6 – GENERAL DISCUSSION AND SUMMARY

6.1 - GENERAL DISCUSSION

In vitro embryo production techniques are rapidly growing and research projects are going on to improve in vitro maturation, fertilization and culture conditions to obtain maximum embryo production, but the success rate is still below 50 %. Application of this technique at the small farms is limited because of the requirement of high technical skills and expensive equipment. High yielding dairy animals, which are selected for particular characteristics by pedigree information and, therefore, have very good genetic material, are often culled after five to six lactations for various reasons with the production of five to six calves. Finding out a low cost proliferation method for the genetic material of these animals will help to increase the reproductive impact of these animals. FSH has been shown to play an important role in the recruitment, selection and dominance of bovine follicular dynamics (Calder and Rajamahendran, 1992; Ginther *et al.,* 1996). It is assumed that the FSH surge, which occurs before each follicular wave stimulates the follicular recruitment and increases the viability of follicles and oocytes. Since it has been shown that a single FSH treatment at the time of estrus increases ovarian response to follicular recruitment (Stubbings *et al.,* 1993), I hypothesized that ovaries removed 2 days after a single dose FSH treatment on the day of standing estrus would stimulate recruitment of more viable oocytes and follicles in the first follicular wave. Collection of oocytes from these follicles on the second day of estrus also reduces the exposure of newly recruited follicles to the suppressive effects of the dominant follicle. In my first

experiment, I showed that FSH priming at the time of standing estrus increased the number of follicles and oocytes recruited for the first follicular wave. Since the first follicular wave emerges around the time of estrus and the newly recruited follicles are not exposed to suppressive effects of the dominant follicle, I expected high viability among oocytes but due to high variation and low sample size I did not get any significant increases in viability. Although I did not obtain a significant increase in oocyte recovery and viability from $PGF_{2\alpha}$ treated non-FSH primed animals, a few studies have indicated that the stage of estrous cycle influences the developmental potential of oocytes (Machatkova *et al.,* 1996). This difference is acceptable because my sample number was small and there was high variability among animals. Because various cryopreservation techniques are available for successful preservation of bovine embryos (Saha *et al.,* 1996; Vajta *et al.,* 1998), the genetic material of culled animals can be preserved long time after successful production of embryos in vitro by combining FSH priming and the ovary slicing technique for oocyte recovery.

It is now possible to transport bovine oocytes within 24 h to most parts of the world. Interestingly, the optimum maturation time for immature bovine oocytes is also 24 h. Determining a convenient maturation method, in which oocytes are transported easily, will be extremely useful for commercial applications. In the second experiment, I matured bovine oocytes in straws because this will ease the transport and recovery of oocytes after maturation. I found that maturing oocytes without 5% CO_2 at 38.5 °C had no effect on subsequent embryo development up to 72 h of culture and had a significant effect on embryo development from 72 h to the 9th day of culture. Similar observations on embryo development up to 72 h after fertilization was reported in one study but in

contrast to my finding, subsequent embryo development was not affected by maturing oocytes without 5% CO_2 (Byrd *et al.,* 1997). Some other studies showed that maturing oocytes without 5% CO_2 reduced embryo development from the beginning (Westhusin *et al.,* 1993). Because of these contradictory results further studies should be carried out to verify whether embryo development up to blastocyst stage is actually affected by maturing oocytes without 5% CO_2.

Recent reports suggested that anti-freeze glycoproteins (AFGP) improved the viability of oocytes after cryopreservation by vitrification and slow freezing in all the mammalian systems tested thus far (O'Neil *et al.,* 1997). Hence, further studies with AFGP and combination of low levels of cryoprotectants may give increased viability of bovine oocytes after slow freezing or vitrification.

6.2 - SUMMARY

In my first experiment, I assessed the number and viability of the oocytes recovered from first follicular wave follicles recruited without any treatment and after FSH priming using a single economical FSH treatment protocol to maximize viable oocyte yield and in vitro embryo production using culled animals. My first experiment revealed that culled cows could be used to produce higher number of embryos.

In my second experiment, I matured oocytes in straws without 5% CO_2 at 38.5 °C without compromising their viability. Although embryo development was significantly lower than the control, I obtained significant results in order to transport oocytes long distance as about 35 % blastocysts could be produced by this method. Some other studies

also showed that 5% CO_2 was not necessary for oocyte maturation. Therefore, I suggest that this method can be used for long distance transport of oocytes.

In my third experiment, I tested two cryopreservation methods with oocytes at two different stage of maturation to find out the ideal method for cryopreservation of bovine oocytes. In the first part of the experiment, I checked the repeatability of one of the best available slow freezing methods for the preservation of bovine oocytes at two different stages of maturation. My results indicated that the repeatability of this slow freezing protocol was very low. In the second part of the experiment, I formulated a new vitrification solution by mixing different cryoprotectants for the vitrification of bovine oocytes. My result showed very poor cryoprotective effect of this new formulation of vitrification solution. Further studies with different combinations of cryoprotectants will verify a suitable cryoprotectant for vitrification.

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APPENDIX 1

Preparation of oocyte aspiration media (modified PBS media)

- Add 0.3gm Bovine serum albumin to 100 ml phosphate buffered saline to prepare 0.3% BSA solution.
- Add 500 µl (10 µg / ul) gentamicin to 0.3% BSA solution, filter it through 0.22 µm Millipore filter and incubate at 38.5 °C in a water-bath for 2 hrs before use.

Preparation of in-vitro maturation medium

- As shown in the table below, prepare the amount of media needed

Ingredient	10 ml	15 ml	20 ml	30 ml	unit(conc)
1. TCM - 199	9.5	14.25	19	28.5	ml
2. Gentamicin	10	15	20	30	µl (50 µg / µl)
3. FSH	10	15	20	30	µl (10 µg / µl)
4. Donor serum	500	750	1,000	1,500	µl

- After preparation of maturation media, sterilize it by filtering through 0.22 Millipore filter.
- Pour the media (2 ml) into small pettri dish and cover it with mineral oil.
- Incubate it at 38.5 °C and 5% CO_2 in the incubator for 2 hrs before use.

APPENDIX 2

1. Preparation of sperm capacitation medium

1.1 Preparation of 500 ml of BO - A solution

1. Sodium Chloride (NaCl)	-	4.3092 gm.
2. Potassium Chloride (KCl)	-	0.1974 gm.
3. Calcium Chloride, Dihydrate ($CaCl_2.2H_2O$)	-	0.2171 gm.
4. Sodium Phosphate, Monobasic ($NaH_2PO_4.2H_2O$)	-	0.0840 gm.
5. Magnesium Chloride Hexahydrate ($MgCl_2.6H_2O$)	-	0.0697 gm.
6. 0.5% Phenol Red ($C_{19}H_{14}O_5S$)	-	0.1 ml.
7. Distilled water upto	-	500 ml.

1.2 Preparation of 200 ml of BO - B solution

1. Sodium Hydrogen Carbonate ($NaHCO_3$)	-	2.5873 gm.
2. 0.5% Phenol Red ($C_{19}H_{14}O_5S$)	-	0.04 gm.
3. Distilled water upto	-	200 ml.

* Pass 5% CO_2 into the solution for 30 min.

1.3 Preparation of 35 ml of BO solution

1. BO - A solution	-	26.6 ml.
2. BO - B solution	-	8.4 ml.
3. Sodium Pyruvate ($C_3H_3O_3Na$)	-	0.0048 gm.
4. Gentamycin	-	35 µl.

1.4 Preparation of 25 ml of Caff - BO solution

1. BO solution	-	25 ml.
2. Caffeine Sodium Benzoate 50 : 50 (w/w) mixture	-	0.0243 gm.

* Sterilize by filtering through a 0.22 um Millipore filter before use.

1.5 Preparation of 10 ml of BO - BSA solution

1. BO solution	-	10 ml.
2. Bovine Serum Albumin	-	0.06 gm.
3. Haparin Sodium (1,000 IU/ml)	-	37 µl.

* Sterilize by filtering through a 0.22 um Millipore filter before use.

1.6 Preparation of Caff-BO-BSA solution for wash the mature oocytes and dilute the semen

1. Caff - BO solution	-	1 part
2. BO - BSA solution	-	1 part

2. Preparation of in-vitro culture medium

* As shown in the table below, prepare the amount of media needed

Ingredient	5 ml	7.5 ml	10 ml	15 ml	20 ml	unit
TCM - 199	4.75	7.13	9.5	14.25	19	ml.
Donor serum	250	375	500	750	1,000	µl.
Insulin	5	7.50	10	15	20	µl.
Gentamycin	5	7..5	10	15	20	µl(50 µg/µl)

- After preparation of culture media, sterilize it by filtering through 0.22 Millipore filter.
- Pour the media (2 ml) into small pettri dish and cover it with mineral oil.
- Incubate it at 38.5 °C and 5% CO_2 in the incubator for 2 hrs before use.