In the name of Allah,

The most merciful,

The most beneficent

Razi University

Faculty of Agriculture
Department of Animal Science

M.Sc.Thesis

Title of the Thesis:

Effect of demecolcine treatment of bovine matured oocytes on further in vitro embryo development

Supervisors:

Dr. Hamed Karami Shabankareh
Dr. Mohammad Hossein Nasr Esfahani

By:

Zeinab Khoobbakht

January 2009

دانشگاه رازی

دانشکده کشاورزی

گروه علوم دامی

پایان نامه جهت اخذ درجه کارشناسی ارشد مهندسی علوم دامی

گرایش فیزیولوژی دام

عنوان پایان نامه:

اثر درمان دمکولسین اووسیت های بالغ شده گاوی بر پیشرفت تکوین رویان آزمایشگاهی

اساتید راهنما:

دکتر حامد کرمی شبانکاره

دکتر محمد حسین نصر اصفهانی

نگارش:

زینب خوب بخت

بهمن ماه ۱۳۸۷

Razi University

Faculty of Agriculture
Department of Animal Science

M. Sc. Thesis

Effect of demecolcine treatment of bovine matured oocytes on further in vitro embryo development

By:

Zeinab Khoobbakht

Evaluated and approved by thesis committee: as Excellent

Dr. Hamed Karami Shabankareh	**Supervisor/ Assist. Prof**
Dr. Mohammad Hossein Nasr-Esfahani	**Supervisor/ Associated. Prof**
Dr. Mozaffar Khazaie	**External Examiner/ Associated. Prof**
Dr. Farokh Kafilzadeh	**Internal Examiner/ Associated. Prof**

January 2009

Acknowledgements

Thank God for giving me the opportunity to learn more in the company of some of his best creatures.

I wish to thank Dr. M.H. Nasr Esfahani for his professional support and guidance. He is the best mentor and professor that any graduate student can have. I appreciated all his encouragements and patience with me.

I'm deeply grateful to my supervisor, Dr. H. Karami Shabankareh, who has adviced me. I consider myself very fortunate for being able to work with a very considerate and encouraging lecturer like him.

I extend thanks to my committee members, Dr. F. Kafilzadeh and Dr. M. Khazaie, for their time, technical expertise, and editing skills.

I expressed extreme gratefulness to Dr. M. Torki, Head of Dept., for helping me in many different ways.

I thank Mr. M. Hajian, Dr. S.M. Hosseini, Dr. M. Foroozanfar, Mrs. P. Abedi and Mrs. L. Hosseini, who helped me enormously. Their assistance during long days of experiments was valuable.

I valued the support of everyone in Royan Institute Isfahan campus: Mr. A.A. Khajeh, Mr. H. Heidari, Mr. M. Shokoohi, Mrs. M. Shakeri and other staff.

I am especially grateful Dr. Sh. Eghbal Said and Dr. M.R. Akhoond, for helping me to statistical analysis.

I'm grateful for my friends in Isfahan: Dr. (Mrs.) Sh. Razavi, Mrs. V. Asgari, Mrs. Kh. Karbalaie, Mrs. S. Ostad Hosseini, Mrs. M. Nematollahi and Mrs. F. Karamali. The kindness, cheer, and prayers that they invested in me have brought refreshment and hope in long and difficult days. Also, I thank all other my friends who never give up in giving their support to me in all aspects of life. From my friends I learned how to receive when people give gladly and how to give goodness to others. Thank you very much my friends, I will never forget all of your kindness.

In addition, I express my heartiest thanks to my parents for their love and affection. They taught me the importance of education and always encouraged me to work harder and better. I thank my lovely and intimate siblings for their moral support.

"Trust in the Lord and do good; dwell in the land and enjoy safe pasture. Delight yourself in the Lord and he will give you the desires of your heart. Commit your way to the Lord; trust in Him and he will do this: He will make your righteousness shine like the dawn, the justice of your cause like the noonday sun. Be still before the Lord and wait patiently for him."

Zeinab Khoobbakht

January 2009

To my parents

Who supported me

To my siblings

Who encouraged me

To my dear friend P. Abedi

To whom I owe a lot

Abstract

Somatic cell nuclear transfer (SCNT) requires removing all genetic materials associated with the chromosomes of recipient oocyte. This Study was designed to further explore the pharmacological role of demecolcine (DM) on assisting enucleation in bovine SCNT. The in vitro matured bovine oocytes were freed from expanded cumulus cells and then denuded oocytes were treated with pronase to dissolve the zona. Zona-free oocytes were then subjected to a 2×2 factorial arrangement to evaluate the effect of two common concentrations (0.05 and 0.4 μg/ml) and durations (30 and 60 min) of DM. Then the oocytes protrusion formation rate were determined in the inverted microscope at various times (0, 3, 6h) post treatment. Treatment of bovine matured oocytes with both concentrations and also at both durations of DM resulted in a protrusion percentage between 60– 80% when oocytes were checked immediately after treatment and no significant differences were detected between the four treatment groups ($P > 0.05$). Furthermore, when DM treated oocytes were evaluated at 3 and 6 hr post-treatment, significantly higher protrusion frequencies were observed in DM 0.4-60 min group (68.2% and 61.4% respectively), but only in comparison to the DM 0.05-30 min group (both times 25%) ($P < 0.05$). Meanwhile, we evaluated the effects of DM on fertilizability and developmental competency of bovine IVM oocytes after being treated with the best effective dosage and duration of DM (Experiment 1). After 23.5 h post oocytes maturation, oocytes were randomly allotted to two treatment groups of DM (0.05 and 0.4 μg/ml for 30 min) and a control group. Cumulus-oocyte complexes (COC's) were then fertilized and cultured in vitro for up to 9 days when the ratios of in vitro embryo development and the viability of the hatched blastocysts were assessed and compared with the control group. The ratios of the cleavage and blastocyst formation of 0.4 (68.6% and 23.3% respectively) and 0.05 DM treated groups (63.5% and 32.8% respectively) were not significantly different from the control group (73.3% and 29.0% respectively) ($P > 0.05$). The results of cell-viability were also not significantly different between the control vs. treatment groups ($P > 0.05$). In conclusion, DM was capable to promote oocyte enucleation and protrusion formation in the cortical region, in concentrations inferior to those used routinely. The reversibility of DM depends on its treatment duration and the concentration used and reduction in treatment duration and concentration of DM may allow better embryonic quality and development rates in bovine oocytes.

Keywords: Demecolcine, Bovine oocyte, protrusion formation, Developmental competency, Viability

List of contents **page**

Chapter 1: Introduction 1

Chapter 2: Literature Review 5
2.1. In vitro embryo production (IVEP) 6
2.1.1. Potential applications of in vitro embryo technologies 6
2.1.1.1. Enhance genetic selection 6
2.1.1.2. Improve fertility 7
2.1.1.3. Optimize breeding schemes 7
2.1.2. In vitro fertilization of mammalian embryos 7
2.1.2.1. Oocyte maturation 8
2.1.2.2. Sperm capacitation 9
2.1.2.3. Oocyte activation and fertilization 9
2.1.3. Production of bovine embryos by in vitro fertilization 10
2.1.4. In vitro embryo culture 11
2.1.5. Embryo production by nuclear transfer 14
2.1.5.1. Factors affecting nuclear transfer efficiency 15
2.1.5.1.1. Recipient cytoplasm 16
2.1.5.1.1.1. Zygotic origin cytoplasm 16
2. 1.5.1.1.2. MII oocytes 17
2.1.5.1.2. Nuclear donors 18
2.1.5.1.2.1. Embryonic blastomeres 18
2.1.5.1.2.2. Embryonic stem (-like) cells 18
2.1.5.1.2.3. Primordial germ cells 19
2. 1.5.1.2.4. Somatic cells 19
2.1.5.1.3. Enucleation 19
2.1.5.1.3.1. Noninvasive enucleation methods 21
2.2. Objective of the study 24

Chapter 3: Materials and Methods 25
3.1. Chemicals 26
3.2. Collection and selection of cumulus-oocyte complexes 26
3.3. In vitro maturation (IVM) 27
3.4. Experimental design 27
3.4.1. Experiment 1 27
3.4.2. Experiment 2 28
3.5. In vitro Fertilization 29
3.5.1. Sperm Capacitation 29
3.5.2. Sperm preparation 30
3.5.3. Determining spermatozoal concentration 30
3.5.4. Calculation of sperm cell concentration 30
3.5.5. Sperm- Oocyte Co culture 31
3.6. Establishment and maintenance of Vero cells monolayer 31
3.6.1. Vero cells thawing 31
3.6.2. Passaging 31
3.6.3. Freezing 31
3.7. In Vitro Embryo Culture 32
3.8. Embryo evaluation 32
3.9. Blastocyst staining 32
3.10. Statistical analysis 32

List of contents **page**

Chapter 4: Results 34
4.1. Induced enucleation of demecolcine-treated oocytes 35
4.2. Effects of demecolcine on bovine embryo development 35
4.3. Evaluation of embryo quality 36

Chapter 5: Discussion 49
5.1. Demecolcine-induced enucleation 50
5.2. Demecolcine reversibility 52
5.3. Effects of demecolcine on bovine embryo development 54
5.4. Effects of demecolcine on bovine embryo quality 55
Conclusion 56

References 57

List of tables **page**

Table 2.1 Non-transgenic somatic cell cloned calves produced using in vitro matured oocytes 17
Table 3.1 Composition of capacitation medium 30
Table 3.2 Composition of Fert-medium 31
Table 4.1 Protrusion frequencies of demecolcine-treated bovine oocytes in different times after treatment 37
Table 4.2 Embryonic development of in vitro fertilized bovine oocytes (Control) 38
Table 4.3 Effect of 0.05 μg/ml demecolcine on embryonic development of in vitro fertilized bovine oocytes 39
Table 4.4 Effect of 0.4 μg/ml demecolcine on embryonic development of in vitro fertilized bovine oocytes 40
Table 4. 5 Effects of different concentrations of demecolcine on embryonic development of and in vitro fertilized bovine oocytes 41
Table 4.6 Cell counts for hatched blastocysts produced from untreated and with two different concentration of demecolcine- treated oocytes 42

List of Figures **page**

Figure 2.1 a schematic representation of the sequence of events that occur during mammalian fertilization.....8
Figure 2.2 Schematic drawing of the bovine acrosome reaction.....9
Figure 2.3 Cortical reaction.....10
Figure 2.4 Individual steps involved in the production of offspring by somatic cell nuclear transfer (SCNT).....16
Figure 2.5 The stages involved in somatic cell nuclear transfer, the transfer of the nuclear DNA from a donor cell into an enucleated unfertilized oocyte.....18
Figure 2.6 Colchicine (a) and demecolcine (b) chemical structures.....22
Figure 2.7 Nuclear transfer procedures (chemical enucleation).....24
Figure 3.1 Evaluation of the quality of oocytes aspirated from antral follicles (2-8 mm in diameter).....27
Figure 3.2 In vitro matured oocyte.....27
Figure 3.3 Experimental design to evaluate the effect of dosage and duration of demecolcine on protrusion formation ratio of in vitro matured bovine oocytes.....28
Figure 3.4 Experimental design to compare the effects of two current concentration of demecolcine on the development of in vitro-derived embryos.....29
Figure 3.5 Hemocytometer and lab counter.....30
Figure 4.1 MII oocytes before (a and b) and after demecolcine treatment (c and d).....43
Figure 4.2 Percentage of protrusion formation in oocytes treated with two different concentrations (0.05 and 0.4 μg/ml) of demecolcine for 30 or 60 minutes.....43
Figure 4.3 The stages involved in induced enucleation of demecolcine-treated oocytes...44
Figure 4.4 Percentage of cleaved IVF-derived bovine embryos that produced from untreated (control) or treated with two different concentrations (0.05 and 0.4μg/ml) of demecolcine.....45
Figure 4.5 Percentage of 8 to 16-cell IVF-derived bovine embryos that produced from untreated (control) or treated with two different concentrations (0.05 and 0.4μg/ml) of demecolcine.....45
Figure 4.6 Percentage of compact IVF-derived bovine embryos that produced from untreated (control) or treated with two different concentrations (0.05 and 0.4μg/ml) of demecolcine.....46
Figure 4.7 Percentage of blastocyst IVF-derived bovine embryos that produced from untreated (control) or treated with two different concentrations (0.05 and 0.4μg/ml) of demecolcine.....46
Figure 4.8 Percentage of hatched blastocyst IVF-derived bovine embryos that produced from untreated (control) or treated with two different concentrations (0.05 and 0.4μg/ml) of demecolcine.....47
Figure 4.9 In vitro development of bovine embryos following demecolcine treatment and counting of the cell numbers of blastocysts after double staining.....48
Figure 4.10 Percentage of viable and dead cells of hatched blastocyst IVF-derived bovine embryos that produced from untreated (control) or treated with two different concentrations (0.05 and 0.4μg/ml) of demecolcine.....48

Chapter 1

Introduction

Nuclear transfer (NT) is one tool for producing cloned animals, i.e. live mammals can be produced without fertilization events. But this does not mean that gametes are not necessary for cloning by nuclear transfer. Actually, successful cloning is in great part dependent on the female gamete – oocyte. Simply to say, nuclear transfer is the technique to replace oocyte chromatin with a foreign nucleus, thus oocyte cytoplasm adopts the new genetic material, then supports further development to term as those events occur as fertilization (Yang, 2005). The technology has been extended to apply to the genetic improvement of farm animals, rescue of endangered species, and production of transgenic animals for medical use and organ transplantation (Tsunoda *et al.*, 2000). Unfortunately, even if cloning is possible in these few experiments, nuclear transfer is a very complex, time consuming, poorly understood and inefficient process (Polejaeva *et al.*, 2000). However, success rates, gauged by the number of live offspring per manipulated embryo, remain in the order of 1%–2% for most domestic species (Kato *et al.*, 1998; Wells *et al.*, 1999). The initial harm caused to the embryo during manipulation and culture apparently takes its toll during the first trimester of gestation, when over 50% of pregnancies originated from NT embryos are lost in cattle, sheep, and goats (Cibelli *et al.*, 1998; Kato *et al.*, 1998; Wells *et al.*, 1999). Efforts to increase the efficiency of this technique have been directed towards improvements in quality of the two basic constituents of NT embryos, i.e., the recipient egg (Bordignon *et al.*, 1998; Peura *et al.*, 1998; Dominko *et al.*, 2000; Liu *et al.*, 2000) and the nuclear donor cell (Renard *et al.*, 2002; Lee *et al.*, 2003), as well as the synchronization between them (Wells *et al.*, 2003). The removal of chromosomes from recipient oocytes is one of the key factors affecting cloning efficiency because of the importance to maintain the correct ploidy. Aneuploidy leads to subsequent detrimental effects on development, genetic interference of the recipient cytoplasm, and possible parthenogenetic activation and embryo development without the participation of a newly introduced nucleus (Dominko *et al.* 2000). Enucleation may also affect the ultrastructure of the remaining cytoplast, thus resulting in a decline or destruction of its cellular compartments (Yang, 2005). To improve the enucleation procedure and thus cloning efficiency, several methods have been attempted.

Unlike mouse and rat whose nuclei can be easily observed and removed under an inverted stage microscope, in some species including rabbit, sheep, goat, cattle, pig and horse, the metaphase zone is difficult to localize. In these species to define metaphase location, it is reasonable to stain the chromosomes with fluorescent dyes in combination with UV irradiation (Yang, 2005). Hoechst 33342, a short-wavelength, UV excitable fluorochrome, is routinely used to label oocyte chromatin Under a fluorescent microscope, metaphase spindle prestained with Hoechst 33342 can be easily removed together with very little surrounding cytoplasm, thereby preserving oocyte volume without compromising enucleation efficiency. Using this enucleation procedure, clones have been produced in several species (Forsberg *et al.* 2002; Kubota *et al.* 2000; Onishi *et al.* 2000; Loi *et al.* 2001). However, UV irradiation has been shown to cause a significant decrease in development of embryos to the blastocyst stage mainly due to induction of alterations of the membrane and intracellular components of the bovine oocytes (Fischer Russell *et al.*, 2005) and of chromatin defects (Dominko *et al.*, 2000). Considering that the oocyte is enucleated during NT, the chromatin defects induced by UV irradiation may not play a role in altering the developmental potential of NT embryos. However, the mitochondrial DNA

remains in the enucleated cytoplast and may be damaged during UV irradiation, altering the metabolism of the reconstructed embryo. Oocyte damage is aggravated by the concomitant removal of a portion of cytoplast surrounding the metaphase plate of the recipient oocyte during the enucleation procedure. This cytoplast is believed to contain mRNA, proteins, and molecular precursors that are essential for early embryonic development until embryonic genome activation (Fischer Russell *et al.*, 2005).

To avoid the damaging effect of UV illumination, enucleation can be accomplished by aspiration of the first polar body and the underlying oocyte cytoplasm in the absence of fluorochromes (Dominko *et al.*, 2000). During this "blind" enucleation, as much as 30% of the oocyte cytoplasm is removed (Bordignon *et al.*, 1998). Dramatic reduction of the oocyte volume leads to a decrease in blastocyst development after nuclear transfer (Dominko *et al.*, 2000) and lower blastocyst cell number (Westhusin *et al.*, 1996). In addition, removal of the cytoplasm directly beneath the first polar body does not always assure the removal of all the chromatin since between 10% (Peura *et al.*, 1998) and 25% (Dominko *et al.*, 2000) or more (Bordignon *et al.*, 1998) of these oocytes may still contain residual DNA. Removal of cumulus cells is required prior to oocyte manipulation, and the denudation process disrupts the connection between the first polar body and the metaphase spindle (Mitalipov *et al.* 1999). Therefore, the first polar body cannot be used as a reliable predictor for the location of the metaphase spindle.

The removal of chromosomes from activated oocytes at the telophase stage is also effective (Ibanez *et al.*, 2003), but decreased maturation promoting factor activity might decrease the viability of nuclear-transferred eggs with somatic cells (Tani *et al.*, 2001).

Some chemicals can be applied to production of enucleated oocytes to be used as recipient cytoplasts for NT procedures. Chemical enucleation is a straightforward approach that causes minimal physical trauma to the recipient oocyte, as opposed to the mechanical preparation of enucleated cytoplasts. Moreover, during the mechanical enucleation of oocytes, 10%–30% of the oocyte cytoplasm is removed along with the spindle, depleting the recipient oocyte of important organelles and cytoplasmic proteins, as well as of spindle-associated factors, that are essential for the maintenance of developmental competence (Simerly *et al.*, 2003). Several drugs have been utilized to chemically induce oocyte enucleation, including etoposide (Elsheikh *et al.*, 1998) nocodazole (Yin *et al.*, 2002) and demecolcine (Baguisi and Overstro¨m, 2000; Gasparrini *et al.*, 2003; Ibanez *et al.*, 2003). Nocodazole treatment of rat MII eggs reportedly stabilizes the metaphase plate as a single and slightly protruding mass (Yin *et al.*, 2002), which enables the mechanical removal of the metaphase cone (Hayes *et al.*, 2001). The effectiveness of nocodazole treatment for removal of the maternal chromosomes and cloning, however, has not been demonstrated (Yin *et al.*, 2002). Demecolcine is a specific microtubule inhibitor that binds to tubulin dimers and prevents microtubule polymerization, thus resulting in the loss of the dynamic spindle microtubules (Baguisi and Overstrom, 2000; Ibanez *et al.*, 2003). Disruption of spindle microtubules by demecolcine impairs chromosome migration, suppresses spindle rotation, inhibits second polar body extrusion, alters chromosome partitioning, and thereby, results in the generation of enucleated oocytes (Ibanez *et al.*, 2003).

Treatment of preactivated oocytes with demecolcine has proven to be effective for oocyte enucleation in the mouse (Baguisi and Overstrom, 2000; Gasparrini *et al.*, 2003; Ibanez *et al.*, 2003), the goat (Ibanez *et al.*, 2002), and bovine (Fischer Russell *et al.*, 2005). Moreover, live cloned mice (Baguisi and Overstro¨m, 2000; Gasparrini *et al.*, 2003) and pig (Yin *et al.*, 2002) have been produced from demecolcine enucleated cytoplasts. However, further studies are needed to better characterize the mechanism of demecolcine-induced enucleation to establish a more efficient protocol for the enucleation of oocytes of various strains and species in nuclear transfer procedures. In addition, because

microtubules play a key role in many cellular processes during embryonic development, the potential side effects of microtubule -disrupting drugs on development of reconstructed embryos must be examined.

In the present study, our aims were to evaluate the effects of dosage and duration of exposure to demecolcine on protrusion formation, in bovine oocytes in metaphase II (MII). In addition, the effects of dosage of exposure to demecolcine on bovine MII oocytes were examined using in vitro fertilization experiments.

Chapter 2

Literature Review

2.1 In Vitro Embryo Production (IVEP)

During natural breeding, the male ejaculate contains millions of spermatozoa, of which only one is required to fertilize each ovulated oocyte. In the absence of mating, spermatozoa in the testis and accessory glands are lysed and phagocytized or lost in urine. Similarly, the ovaries of fetal cattle contain about 3 million germ cells. Most of these undergo atresia, with about 200,000 (7%) remaining in the newborn calf. Still less than 1% of the germ cells present at birth are ovulated. Even among the gametes that are released/ovulated, only a fraction participates in fertilization and subsequent development. Although artificial insemination and/or embryo transfer can be applied to some conditions, problems may be managed using the more recently developed embryo technologies (Gordon 1994; Hansel 2003). The reasons for developing systems for in vitro embryos production of farm mammals were for the production of a large number of genetically superior offspring and the production of transgenic animals (Glaser, 2007).

2.1.1 Potential applications of in vitro embryo technologies

2.1.1.1 Enhance genetic selection

In vitro embryo technologies have great potential for improving the rate of genetic gain for quantitative traits important for meat and milk production. Both the intensity and accuracy of selection for quantitative traits can be improved through the use of in vitro embryo production (Hansen and Block, 2004). In addition, in vitro embryo production systems can reduce the generation interval through the production of embryos from pregnant animals (Block, 2007) and there is also the promise of producing embryos from prepuberal heifers, although problems with oocyte competence need to be addressed (Salamone *et al.*, 2001). Further improvements in genetic gain may also be possible since recent reports indicate that oocytes can be produced from stem cells (Kehler *et al.*, 2005) or derived from cells present in bone marrow (Johnson *et al.*, 2005). Such procedures may eventually allow for an unlimited pool of oocytes from genetically superior females (Block, 2007).

There is also potential to optimize genetic selection through the application of in vitro embryo technologies. The use of techniques for preimplantation genetic diagnosis (Bredbacka, 2001; Moore and Thatcher, 2006) can allow for selection of embryos based on their specific allelic inheritance prior to transfer. Several genetic markers have been identified, including markers for milk production traits (Spelman *et al.*, 2002; Freyer *et al.*, 2003), growth and carcass traits (Casas *et al.*, 2000) and recently, fertility (Garcia at al., 2006). Another genetic trait that is of great importance, particularly in the dairy industry, is sex. While embryo sexing using the polymerase chain reaction has become very common in the commercial embryo transfer industry (Lopes *et al.*, 2001), the advent of sexed semen technology (Seidel, 2003) provides another strategy for skewing sex ratio that has many potential applications in cattle production (Hohenboken, 1999). In vitro embryo production systems provide a more practical approach for the use of sexed semen because more embryos can be produced with sexed semen in vitro than by using superovulation (Bousquet *et al.*, 1999)

2.1.1.2 Improve fertility

The fertility of lactating dairy cattle has declined over the past 40-50 years (Lopez-Gattius, 2003). While the causes of infertility are not fully understood, lactation is associated with reduced oocyte competence (Snijders *et al.*, 2000) and poor early embryo development (Sartori *et al.*, 2002). These problems could potentially be bypassed through the use of in vitro embryo transfer. The use of oocytes collected from abbatoir-derived ovaries can be used as an inexpensive source of genetic material for producing embryos for large scale embryo transfer breeding schemes. While there are producer concerns about the genetic make-up of abbatoir-derived oocytes, a study by Rutledge (1997) indicates that the genetic merit of cows sent to slaughter is only slightly lower than for the average cow in the herd of origin. Moreover, the ability to produce hundreds of embryos with only a few straws of semen allows for genetic improvement by utilizing semen from genetically valuable sires that in other instances, would be too expensive. To date, few studies have directly compared the pregnancy rates obtained with artificial insemination versus embryo transfer in lactating dairy cattle (Al-Katanani *et al.*, 2002). The use of embryo transfer in situations where pregnancy rates to artificial insemination are above average does not seem to provide any increase in fertility (Sartori *et al.*, 2006). In contrast, however, in cases where pregnancy rates to artificial insemination are low, such as during heat stress, in vitro embryo transfer can be effective in improving fertility in lactating dairy cows (Al-Katanani *et al.*, 2002).

2.1.1.3 Optimize breeding schemes

Production of embryos in vitro also has potential for enhancing crossbreeding schemes. The use of crossbreeding to take advantage of heterosis is commonly used in beef production systems. While seldom used for dairy cattle production in the United States, crossbreeding has received renewed attention recently (McAllister, 2002; Heins *et al.*, 2006a; Heins *et al.*, 2006b). Production of F1 crossbred embryos in vitro for transfer to F1 recipients could improve crossbreeding schemes by eliminating the loss of heterosis and increased phenotypic variation that occurs when F1 females are mated to purebred or crossbred sires (Rutledge, 2004).

More than 50% of the costs associated with beef production are derived from maintenance of the mother cow in single calving herds. The use of embryo transfer to induce twinning in beef cattle could be important for increasing the efficiency of beef production (Block, 2007). Although induced twinning is not routinely used in beef cattle production systems, the precipitous decline in land for agriculture use and continued population growth may necessitate the use of such schemes in the future. As with producing embryos in vitro to mitigate problems of infertility in dairy cattle, the production of embryos in vitro for induced twinning represents a more practical alternative compared to superovulation. There is also potential for in vitro embryo production and induced twinning in dairy production systems. A recent study incorporated in vitro embryo transfer with sexed semen and induced twinning in beef cattle to produce Holstein heifers as replacements for dairy operations (Wheeler *et al.*, 2006)

2.1.2 In vitro fertilization of mammalian embryos

Fertilization encompasses a series of events that result in the fusion of the male and female gametes, specifically: (1) contact with and penetration of the cellular investments of an oocyte by a spermatozoon; (2) penetration of the zona pellucida; (3) fusion of the spermatozoon and oocyte external membranes, and (4) pronuclei fusion (syngamy) and alignment of their respective chromosomes on the first cleavage spindle (Figure 2. 1). Ultrastructure studies have documented each of these physiological events. (Hafez *et al.*, 2000).

The process of mammalian in vitro fertilization (IVF) is the joining together of a male and female gamete outside of the body (Glaser, 2007). Recorded attempts at in vitro fertilization date back to 1878. However, M.C. Chang was the first to unequivocally demonstrate successful in vitro fertilization followed by the birth of live offspring (Bavister 2002). Since then, similar successes have been reported in a number of species. In vitro fertilization, with subsequent production of offspring after the transfer of resulting embryos to recipient females has been reported in at least 30 domestic and nondomestic mammalian species. For fertilization to occur the medium conditions must be optimal. There are a number of events that must take place before an embryo develops, resulting in implantation (Wirtu, 2004).

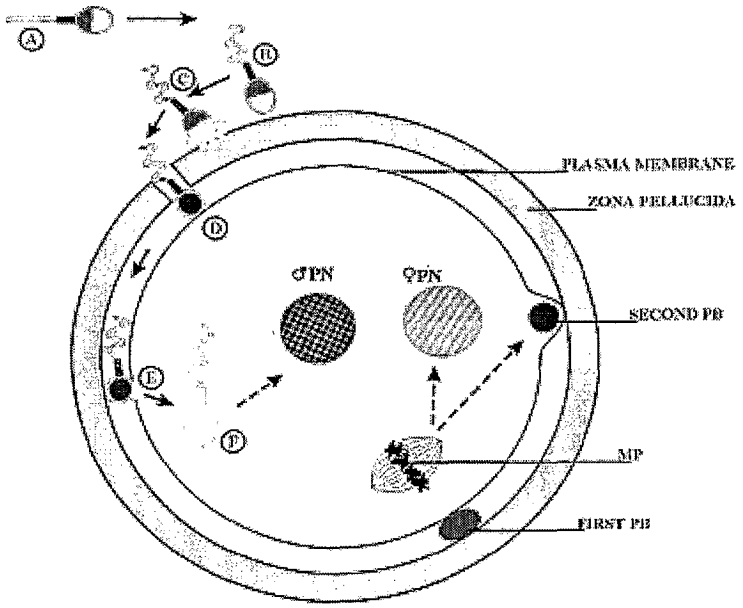

Figure 2.1 a schematic representation of the sequence of events that occur during mammalian fertilization. Inside the female genital tract, spermatozoa are activated during a process called capacitation (A). Capacitated sperm cells become hypermotile and able to bind to the zona pellucida (B) and thereby trigger the acrosome reaction (C). The hydrolytic enzymes thus released lyse the zona pellucida (D) and enable the hyperactive spermatozoon to enter the perivitelline space, where it can bind to the oolemma (E), fuse with and become incorporated into the oocyte. Thereafter, the sperm head begins to swell (F) and the oocyte, which was arrested at metaphase of the second meiotic division (MII) with its chromosomes arranged along the metaphase plate (MP), is activated and progresses through meiosis to extrude the second polar body. Finally, the female and male pronuclei are formed as the final prelude to syngamy (Tremoleda, 2003).

2.1.2.1 Oocyte maturation

For fertilization to occur an acrosome reacted spermatozoan (sperm) enters a matured oocyte. During oocyte maturation there is progression of nuclear maturation from the germinal vesicle of the arrested dictyate stage, through meiosis I to metaphase II and extrusion of the first polar body (First and Parish, 1987). There are a few key factors that are important for complete oocyte maturation to occur. Moor and Gandolfi (1987) indicated that especially important are the development of proteins in the cytoplasm which regulate: meiotic events, sperm decondensation, formation of the male pronuclear envelope, initiation of cleavage, completion of cleavage-stage, embryo development and the ability to produce a viable offspring.

All of these regulatory events can be affected by the duration of the in vivo or in vitro maturation time of the oocyte and the culture conditions during in vitro maturation. Common defects resulting from fertilization of immature oocytes include failure of male

pronuclear development and failure to progress to organized blastocyst stage embryos (Glaser, 2007).

2.1.2.2 Sperm capacitation

Sperm must also go through a maturation process known as capacitation before the male gamete is able to fertilize an oocyte. This process normally takes place in the female reproductive tract. However, in the in vitro environment a culture condition must be recreated similar to that of the environment in vivo. Capacitation consists of two main functions, an initial sperm membrane alteration, which allows the sperm to undergo the second phase, the acrosome reaction (Figure 2. 2). In mammalian species, sperm must begin to penetrate the cumulus and corona radiata with an intact acrosome. There is a release of enzymes from the acrosome of the sperm, such as hyaluronidase, starting the digestion of proteins in the zona pellucida enabling fertilization. The acrosome reaction of the sperm is then stimulated by the interaction of the contact between the sperm and the zona pellucida. Fusion of the plasma membrane occurs with the outer acrosomal membrane vesiculation thus, exposing the acrosome contents (Glaser, 2007).

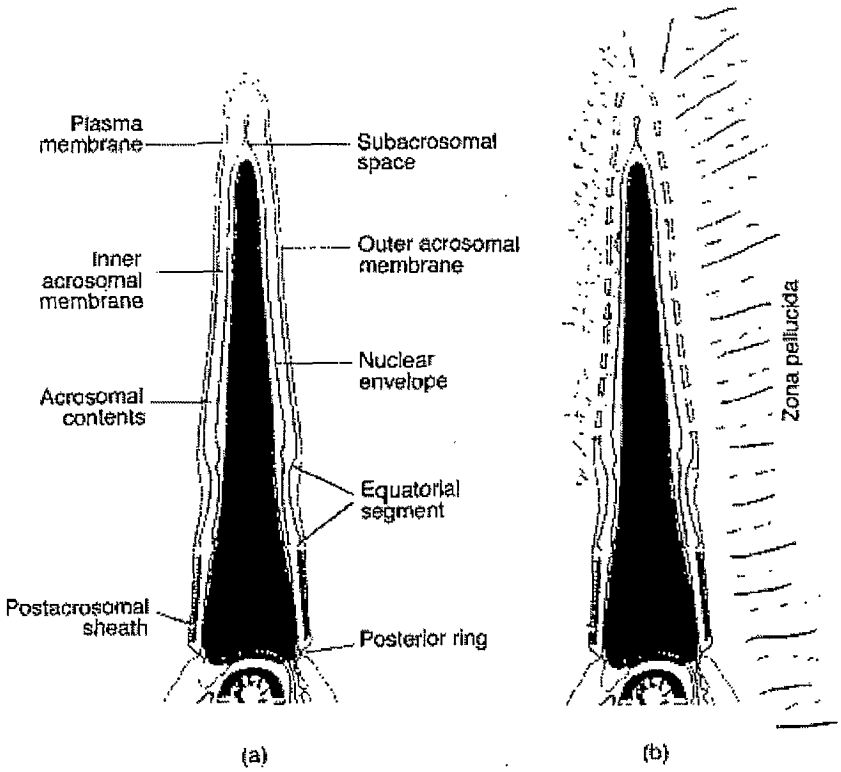

Figure 2.2 Schematic drawing of the bovine acrosome reaction: (a) intact sperm; (b) sperm during fusion of the plasma and outer acrosomal membranes (Gordon, 2003).

2.1.2.3 Oocyte activation and fertilization

In mammalian species, once the first sperm binds to the zona pellucida activation of that oocyte occurs. To prohibit polyspermy, penetration of the zona by more than one sperm, the oocyte responds with a cortical reaction (Figure 2. 3). This cortical reaction produces a block to polyspermic fertilization. There is a massive exocytosis of cortical granules containing a mixture of enzymes released into the perivitelline space, which causes the zona pellucida to harden. Also the sperm receptors are lost preventing additional sperm from binding to the zona pellucida (Glaser, 2007).

Once the individual sperm penetrates the zona pellucida, the sperm nucleus decondenses in the cytoplasm of the oocyte, resulting in the swelling of the sperm head