DISSERTATION

# EFFECTS OF ROSCOVITINE ON IN VITRO MATURATION OF EQUINE OOCYTES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LILIAN CLARION FRANZ ENTITLED: "EFFECTS OF ROSCOVITINE ON IN VITRO MATURATION OF EQUINE OOCYTES" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREEE OF DOCTOR OF PHILOSOPHY (PhD).

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ABSTRACT OF DISSERTATION

# EFFECTS OF ROSCOVITINE ON IN VITRO MATURATION OF EQUINE OOCYTES

Roscovitine is a potent inhibitor of Maturation Promotion Factor (MPF) due to its kinase inhibiting mechanism, competing with ATP for its binding site. This effectively maintains oocytes of several species in the germinal vesicle stage of meiotic arrest without decreasing the developmental potential of embryos that result after removal of roscovitine and subsequent fertilization. The first series of experiments was pursued to determine the effective dose of roscovitine for maintaining equine oocytes in the germinal vesicle stage (GV) and its reversibility after release of inhibition. Ovaries were obtained from an abattoir, and follicles ranging between 5-22 mm in diameter were scraped with a bone curette. Recovered oocytes were classified based on surrounding cumulus cells as compact or expanded, and maturation was initiated during transport to the laboratory in a portable incubator. EMM1 (a synthetic oviductal fluid, plus amino acids) was used without additives as a base medium. Roscovitine was added at 22, 66 or 200 μM for the first experiment. LH, FSH, estradiol 17-β, progesterone, EGF, IGF-I, and fetal bovine serum were added to the base medium as controls, and all oocytes were incubated for 30 h. For oocytes with expanded cumulus, 7, 38 and 55% remained in the GV stage with 22, 66, 200 μM roscovitine treatments; for oocytes with compact cumulus, 45, 73 and 56% remained in the GV stage with 22, 66, 200 μM roscovitine treatments. For Experiment 2 of the first series, oocytes were incubated with 66 μM roscovitine for

24 or 32 h followed by an additional 24 h in control medium. MII rates after inhibition and release were similar for all treatment groups and oocyte types, including the control group (mean=72%). The third part of the first series evaluated maturation promoting factor (MPF) activity, using a histone kinase assay of oocytes matured with or without 66 μM roscovitine. A 32 kd phosphorylated band was present in all categories of oocytes incubated in control medium at 16, 30 and 48 h of maturation. Oocytes with expanded cumuli that were incubated with roscovitine for 24 or 32 h and then for additional 24 h in control medium had a band of similar size and density as the oocytes in control medium for 16, 30 and 48 h; oocytes with compact cumulus cells had lower MPF activity ($P<0.05$) when incubated with roscovitine compared to controls.

The second study concerned developmental potential of equine oocytes after culture with 66 μM roscovitine for 24 h. Within 90 min of slaughter, oocytes were placed in either EMMI with 66 μM roscovitine and BSA or control medium with LH, FSH, estradiol 17-β, progesterone, EGF, IGF-I, and fetal bovine serum. A subset of oocytes in the roscovitine treatment was fixed and stained at the end of 24 h of culture; of these, 26/31 (84%) of the oocytes with compact cumulus cells and 16/28 (57%) of the oocytes with expanded cumulus cells had a germinal vesicle. Three culture groups were evaluated: maturation after 30 h in control medium; 24 h in roscovitine medium, and then 30 h in control medium; and 54 h in control medium. There were no differences between maturation rates to metaphase II among the treatment groups and cumulus types. After culture, oocytes with a first polar body were subjected to intracytoplasmic sperm injection (ICSI) using a piezzo drill, and then cultured in G1/2 media for 96 h, assessed for morphological cleavage, and stained with Hoechst 33258 to visualize nuclei. No

differences were detected in morphological cleavage rates after ICSI among treatments within cumulus type: percentages of oocytes with compact cumulus cells cleaving after 30 h in control medium, 24 h in roscovitine plus 30 h in control medium, and 54 in control medium were 82, 80, and 83%, respectively; for oocytes with expanded cumulus cells 76, 71, and 62%, cleaved respectively. Significantly more oocytes with compact cumulus yielded embryos having $\geq$ 8 nuclei in the groups incubated with roscovitine first and then transferred to control medium and those that were cultured for 30 h in control medium (63 and 50%) than for 54 h in control medium (8%; $P < 0.01$). For oocytes with expanded cumulus cells, the proportion of embryos with $\geq$ 8 nuclei was higher in oocytes cultured for 30 h in control medium (44%) than for 54 h in control medium (8%; $P < 0.05$), and higher for oocytes incubated with roscovitine for 24 h and then for 30 in control medium (35%) than for 54 h in control medium ($P = 0.08$). The average number of nuclei/embryo was higher in oocytes with compact cumulus cells that were incubated with roscovitine for 24 h and then for 30 h in control medium ($13.5 \pm 2.1$) than in any other group ($3.6 \pm 0.9$ to $9.3 \pm 1.4$, $P < 0.05$). These results demonstrate that roscovitine can be used to maintain equine oocytes in the germinal vesicle stage for up to 24 h without decreasing their developmental potential. This treatment with oocytes of compact cumulus optimized development of resulting embryos.

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# PREFACE

## PhD: Doctor of philosophy

Philosophy: 1. The inquiry into the most comprehensive principles of reality in general, or of some limited sector of it such as human knowledge or human values.

2. Practical wisdom; fortitude, as in enduring reverses and suffering.

Fortitute: strength of mind in the face of pain, adversity, or peril, patient and constant courage.

**(The Readers Digest great encyclopedic dictionary 3rd edition, 1969)**

## Thesis is an idea, dissertation is the document that supports your thesis (Olin Shivers)

*Doing a PhD is more than exploring a subject in all of its depth. My experience in graduate school exceeds the introduction to the scientific world (once so distant and fascinating to me): it was the most interesting process of self-awareness. No other program, or life style, is so efficient to make one learn about her or himself and her (his) inner most profound needs as a human being.*

*Here, I present my dissertation entitled "Effects of roscovitine on in vitro maturation of equine oocytes". Despite my humble abilities as a writer, I hope that my point of views are not unique and that work here introduced will contribute with science and the emerging technology. Foremost, it was done with enthusiasm and gratitude.*

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Half a decade as a young adult, I spent seeking knowledge at graduate school. For every question, each project, and every insight made from a mistake, a number of different people were involved. Some for a short span of time, some for a long period, some intense, some superficial... all indispensable.

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The experiments in this dissertation involved three well-known institutions in the equine reproduction world: Colorado State University, Embrapa-Cenargen, and Texas A&M University. Working with each was a privilege. I would like to thank the benefactors of the Preservation of Equine Genetics (PEG) program and the Lucy Whittier foundation for providing funding for all experiments at CSU, and to Embrapa for giving me all the logistic support to accomplish the experiments conducted in Brasil, and also to the Horse endowment link fund at Texas A&M.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

Despite constant progress in improving embryo production in vitro in horses, the overall yield of these techniques remains unsatisfactory, due to low in vitro fertilization rates and poor development of zygotes produced in vitro. The common denominator of this equation is the oocyte and its maturation process. Maturation is the last step of a long differentiation process in which the oocyte acquires ability to be fertilized and to support embryonic development (Mermillod et al, 1999). Completion of nuclear maturation, which culminates with extrusion of the first polar body and formation of a metaphase II plate, is not equivalent with developmental competence, nor does it reflect the molecular and structural maturity of an oocyte (Trounson et al, 2001).

The nuclear competence for meiotic maturation occurs earlier than developmental competence to form an embryo (Arlotto et al, 1996, Blondin et al, 1995, Fair et al, 1995), and final oocyte maturation requires complete cell biological machinery to support fertilization and early embryonic development (Hyttel et al, 1997). In vivo, follicles grow and oocytes are maintained in the germinal vesicle stage (GV) by the follicular environment until the preovulatory stage, which in vertebrates culminates with the surge of gonadotropins. By removing the young oocyte from the inhibiting environment of the follicle, it will resume meiosis (Pincus and Enzmann, 1935), as occurs in in vitro systems, but the cytoplasm may not be adequately matured to sustain normal embryonic development (Hendriksen et al, 2000). Recently, more attention has been paid to the

maturation occurring in the oocyte's cytoplasm and its correlation to developmental competence. This is relevant because conventional IVF has had limited success in horses, with only two foals born more than a decade ago (Palmer et al, 1991, Bezard et al, 1992), both from an oocyte matured in vivo which was placed back into the oviduct soon after presumptive fertilization. Despite in vitro nuclear maturation rates greater than 65% (Dell'Aquila et al, 1995; Dell'Aquila et al, 1997), and fertilization rates around 33% (Dell'Aquila et al, 1996), cleavage rates with standard IVF in the equine are is still quite low: 14% to 24% (Dell'Aquila et al, 1997, Zhang et al, 1990; respectively). Therefore, the concept of allowing cytoplasmic maturation while nuclear maturation is blocked in vitro would mimick the final steps of differentiation that occur in late folliculogenesis, and might lead to better developmental competence (Hendriksen et al, 2000). Synchronizing nuclear and cytoplasmic maturation can be achieved with physiological methods (follicular fluid, granulosa cells, thecal cells, follicle hemisections) or pharmacological agents (Dode et al, 2001) such as protein synthesis inhibitors (Lonergan et al, 1997; Tatemoto et al, 1994) and phosphorylation inhibitors (Saeki et al, 1997; Motlik et al, 1990). Although very effective, some of these pharmacological agents result in loss of developmental potential at the end of the treatment (Lonergan et al, 2000).

Recently, roscovitine, a purine-analog that specifically inhibits maturation promotion factor (MPF) kinase activity (Marchal et al, 2001), has been used successfully to maintain bovine oocytes in the GV stage temporarily without compromising further development of the embryos (Mermillod et al, 2000). In the horse, meiotic inhibitors such as cycloheximide and 6- DMAP also were effectively used to maintain GV in a reversible

manner both in oocytes within compact and expanded cumulus cells (Alm et al, 1996; Hinrichs et al, 2002). Roscovitine was also effective in suppressing meiosis in equine oocytes, but appeared to have differential effects depending upon oocyte type (Hinrichs, 2002).

The most recent studies on equine intracytoplasmic sperm injection (ICSI) have reported good activation and cleavage rates (Choi et al, 2002a; Galli et al, 2002). Therefore, ICSI has become an important tool to study developmental competence of oocytes after in vitro maturation, since it bypasses several steps of the fertilization process that still are poorly understood in the horse. Nevertheless, reports on production of embryos after ICSI are few (Squires et al, 1996, Dell'Aquila et al 1997; Galli et al, 2000; Li et al, 2000; Maclellan et al 2000) with five live foals produced to date (Squires et al, 1996, Cochran et al, 1998 and McKinnon et al, 1998).

The experiments in this dissertation were conducted to:

1) evaluate the effectiveness of roscovitine in maintaining equine oocytes in the GV stage

2) determine the ideal inhibition time and dose of roscovitine

3) assess the reversibility of this effect in nuclear maturation

4) analyze the developmental potential of oocytes inhibited after ICSI

# CHAPTER II

# REVIEW OF THE LITERATURE

## OOCYTE MATURATION

Maturation is the last step of a long differentiation process in which the oocyte acquires ability to be fertilized and to support embryonic development. It includes the completion of two cellular programs: nuclear maturation (resumption of meiosis from the GV stage to the second metaphase stage) and cytoplasmic maturation (molecular and structural changes in the mature egg to support fertilization and early embryonic development) (Bevers et al, 1997).

### *Initial oocyte development*

The formation of the female gamete begins in the mammals during early embryogenesis. Primordial germ cells differentiate into oogonia, which multiply by mitosis. The fetal ovary in larger mammals has millions of oogonia, which divide by mitosis into primary oocytes that started the first meitotic cell division, and become primary oocytes enclosed within the primordial follicles. (Austin et al, 1973). This transformation is accompanied by nuclear changes, such as DNA replication (similar to the S-phase in mitotic cells). The oocyte enters the dictyate (late prophase of meiosis) with chromosomes highly diffuse and surrounded by a nuclear membrane (germinal vesicle stage) (Homa et al, 1995). The oocyte remains at this stage, even during continued follicular growth, and does not

resume meiosis until puberty under the influence of a gonadotropin surge (Mattioli et al, 1998).

In vivo, with follicle growth, the oocyte increases in size and, as a consequence of paracrine and endocrine effects, it achieves the ability to complete the first meiotic division, with the extrusion of the first polar body (Sirard et al, 1998).

Concomitant with oocyte growth, the surrounding somatic cells proliferate, and the flattened granulosa cells become cuboidal, enclosing the germ cell in several layers; once there is a solid multilaminar structure, the antral cavity is formed in response to gonadotropin regulation (Buccione et al, 1990). The initial phases of follicular growth and pre-antral development occur independently of gonadotropic hormones.

***Cell coupling and inhibition of meiosis***

In primordial and primary follicles, communication between the oocyte and the granulosa cells is mediated by an endocytic pathway as signaled by abundant coated pits and vesicles. In the secondary and subsequent follicle stages, communication is switched to gap junctions (Hytell et al, 1997). The follicle produces several inhibitory substances synthesized in the cumulus cells and transferred to the oocytes via gap junctions that maintain the oocyte in meiotic arrest (Eppig and Downs 1984; Bilodeau et al, 1993).

Substances produced by the follicle that inhibit oocyte maturation have been identified: oocyte maturation inhibitor (OMI) (Tsafriri et al, 1982), β-

endorphins (O et al, 1990) and Mullerian inhibiting substance (MIS) (Takahashi et al, 1986). In mice hypoxanthine, which originates from thecal cells, has been identified as the principal molecule that inhibits maturation, (Downs et al, 1985). Other purines involved in inhibition are guanosine and adenosine (Eppig et al, 1985; Salustri et al, 1988, Hubard et al, 1982) and cAMP (Sirard et al, 1998), which is by far the most studied. In general it is accepted that a high level of cAMP within the oocyte maintains meiotic arrest whereas a decrease of cAMP levels precedes meiotic resumption (Downs, 1993). It has been suggested that closure of the gap junctions terminates the passage of the inhibitory signal (Dekel et al, 1978). Controversially, intra-oocyte levels of c-AMP do not necessarily decline during meiotic resumption (Homa, 1995). Therefore, one or more factors can override the inhibitory effect of cAMP (Eppig, 1991, 1993).

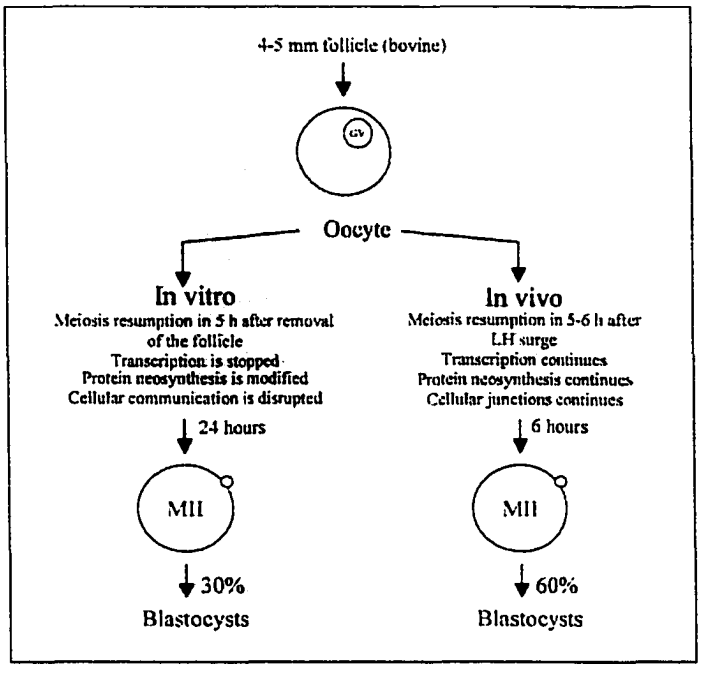
***Release of inhibition***

A preovulatory surge of LH induces meiotic maturation but oocytes are devoid of LH receptors (Lawrence et al, 1988), indicating the importance of the somatic cell component. After the surge of gonadotropins (LH), the role of the follicular cells switches from generating inhibitory signals to that of giving support to the maturing oocyte (Bevers et al, 1997). Therefore, for maximal developmental potential, the integrity of the follicular support must be preserved (Kito et al, 1997). As a result of the LH/FSH surge, the cumulus cells expand due to the production of hyaluronic acid.

Cumulus expansion facilitates the detachment of the oocyte from the follicle and its capture by the fimbria of the oviduct upon ovulation. The support of the cumulus cells plays an important role in the process of maturation since metabolites, and regulation factors pass through the gap junctions between the cumulus and the oocytes. With expansion of the cumulus cells, the connections to the oocyte are compromised, and cAMP levels decrease. Resumption of meiosis is stimulated by LH and is mediated through calcium via the cumulus cells. While levels of intracellular calcium normally increase in cumulus-intact oocytes, meiotic resumption diminished when calcium concentration was reduced in the medium. The loss of oocyte viability due to calcium depletion has been reported in the rat and cow, but it is lethal in mouse (Homa et al, 1995) and cow oocytes (Keller et al, 1993). Direct transport of calcium through the inositol-triphosphate is followed by a calcium-induced calcium release in the oocyte itself (Homa, 1993).

Release of the cumulus oocyte complex from its follicular environment spontaneously induces the resumption of meiosis, hastening the events in the maturation process, similar to what occurs systems in vitro. In Figure 1, maturation resumption in vivo and in vitro in bovine oocytes is compared. Maturation in vitro is initiated by removal of oocytes from the follicle and takes 24 h whereas in vivo oocytes will resume meiosis 6 h after the LH surge in bovine oocytes.

Figure 1- Comparison between in vivo and in vitro maturation in bovine oocytes




Source: Mermillod et al, 1999.

***Hormonal control***

Prior to the burst of granulosa cell proliferation to form a preovulatory follicle, granulosa cells acquire enhanced responsiveness to FSH and LH via synthesis of their receptors and begin producing estradiol 17-ß (Robker et al, 1998). FSH receptors are present in granulosa cells from the beginning of antral follicular development. In response to FSH, granulosa cells divide and synthesize estradiol 17- ß, which enhances its own expression. Then both FSH and estradiol 17-ß promote granulosa cell division and induce additional LH receptors. Once induced, the LH receptors require continuous presence of FSH for their maintenance (reviewed in Romero-Arredondo, 1991). Upon stimulation of FSH

and LH receptors, cAMP levels increase in the oocytes (Crosby et al, 1985; Dekel et al, 1988). In mice, treatment with FSH delays spontaneous meiotic resumption about 3 h due to an increase in the cAMP in oocytes surrounded by cumulus cells; this was not observed in denuded oocytes (Salustri et al, 1985). FSH also stimulates production of a meiosis activating substance (MAS) that is secreted by the cumulus cells and transferred to cumulus intact oocytes (Byskov et al, 1997). Growth factors such as epidermal growth factor (EGF) and insulin growth factor-I (IGF-I) have been isolated from follicular fluid of several mammalian species with levels ranging between 2 to 15 ng/ml (Lonergan et al, 1996). These are synthesized by the ovary, and their effects on regulation and modulation of maturation are mediated via granulosa and or cumulus cells in vivo (Dekel et al, 1985) or directly to the oocyte in denuded oocytes in vitro (Im et al, 1995). EGF receptors have been isolated from ovarian tissue of several species such as bovine (Pohland et al, 1994), pigs (Singh et al, 1995), humans (Maruo et al, 1993) and rats (Chabot et al, 1986). In the horse, EGF receptors have been identified in the follicle and granulosa cumulus cells (Lorenzo et al, 2001), and in bovine the highest expression of EGF receptors was found in the preovulatory follicle (Lonergan et al, 1996). EGF and IGF increase the number of receptors for LH in the granulosa cells (Lorenzo et al, 1995). Moreover, EGF stimulates granulosa cell proliferation and modulates steroidogenesis in granulosa cells (Hsueh et al, 1981). The positive effect of FSH, EGF and IGF-I in follicular growth has recently been described in a long term in vitro culture system of bovine follicles, similarly to what is believed to occur in vivo (Gutierrez et al, 2000). The use of

EGF in in vitro maturation systems increases cytoplasmic maturation in pigs (Abeydeera et al, 1998) and cattle (Im et al, 1995). Lorenzo et al, (1994), demonstrated that the use of EGF and IGF-I combined stimulate nuclear maturation in cattle oocytes more than either of the growth factors alone. Romero-Arredondo et al (1991), detected a molecule in bovine follicular fluid with molecular weight of >10,000 that stimulated meiosis and induced cumulus expansion. This molecule was present in the follicular fluid as early as 8 h after the LH surge. Initially, it was hypothesized that it was EGF, but the MW of EGF is 7000.

In summary, the process of oocyte maturation is complex and involves several factors. The resumption of meiosis in vivo can be explained both by a decrease of gap junctions between cumulus cells and oocyte and also by stimulators produced in the follicle which overcome the inhibition (Downs, 1998).

***Oocyte maturation in vitro***

Most in vitro maturation systems were based on knowledge that oocytes will resume meiosis (nuclear maturation) when removed from the follicular environment. Results of in vitro maturation, based on visualization of a normal metaphase plate with a polar body, have been published in all domestic species, including the horse. Unfortunately, the regulation of in vitro maturation of oocytes cannot be extrapolated from the events occurring in vivo (Bevers et al, 1997). It is well known that the processes in vitro are hastened when compared to in vivo: germinal vesicle breakdown in cattle, for example, occurs in vivo after

the LH surge and takes 7 to 10 h, while in vitro it only takes 5 to 6 h after removal of oocytes from the follicle (De Loos et al, 1991, 1994, Hyttel et al, 1997).

The importance of follicle size has been well documented. Smaller follicles simply do not possess all the elements to produce offspring in vitro in a repeatable manner due to differentiation at the more advanced stages of follicular development (Hendriksen, 2000). Therefore, follicle size greatly affects the ability of the oocyte to mature and develop to the blastocyst stage in vitro. Lonergan et al, (1994) reported that 66% of bovine oocytes developed to blastocysts from follicles > 6 mm in diameter, but only 32% blastocysts for oocytes from follicles 2-6 mm in diameter. In the horse, follicles > 12 mm in diameter enclose more competent oocytes (Hinrichs et al, 2000). In the cow and pig, oocytes derived from follicles > 3mm reach MII and produce blastocysts in vitro at a higher percentage than follicles < 2mm (Motlik et al, 1984; Mattioli et al, 1988; Lonergan et al, 1994). The metaphase II stage coincides with the time most protein synthesis is completed (Fair et al, 1995). Differences such as the expression of LH receptors by granulosa cells (Bevers et al, 1997) and major changes in the follicular fluid have been reported (Collins et al, 1997). The diameter of the oocytes within follicles of the same size vary considerably (Arlotto et al, 1996; Fair et al, 1995), suggesting that follicles in the same size range are not necessarily in the same stage of development. Nevertheless, small follicles have been used in maturation protocols with some success, but instead of following the processes of a preovulatory follicle, maturation-like changes are induced by atresia (Hyttel et al, 1997; Sirard et al, 1998). Morphological changes

are also observed such as cumulus expansion observed in vivo is much more evident than in vitro (Hyttel et al, 1986). It is not surprising that in the bovine for example, higher blastocyst rates are observed with oocytes from preovulatory follicles than small ones (52% vs, 30%). Similarly, in the mare, oocytes from preovulatory follicles resulted in high pregnancy rates after oocyte transfer, while only 9% pregnancy rates were achieved with oocytes matured in vitro (Scott et al, 2001).

***Oocyte capacitation***

The ultrastructure of oocytes that reach the preovulatory stage has been described. Changes occur such as a decrease in the size of the Golgi complexes and more superficial location of cortical granules, undulation of the nuclear membrane, vacuolization of the nucleolus, increase in the perivitelinic space and retraction of the processes of the corona cells into the zona (Hyttel et al, 1997). Most of these changes occur just prior to the LH surge, and they have been characterized as oocyte capacitation or pre-maturation. Interestingly, some of the same changes occur in oocytes undergoing atresia (Assey et al, 1994). Therefore, better in vitro results for cattle have been achieved when ovaries were kept for 4 h before oocytes were harvested and this was attributed to the initial atresia induced in follicles by this procedure (Blondin et al, 1997).

The objective of IVMFC is to produce healthy offspring. Therefore, more attention has been paid to the cytoplasm of the oocyte. It is believed that greater efforts to synchronize cytoplasmic and nuclear events are required for oocytes

originating from non-preovulatory follicles, since oocytes matured in vitro are deprived of prematuration events, negatively affecting their competence in vitro (Hendriksen et al, 2000).

***Indicators of cytoplasmic maturity***

Markers for cytoplasmic maturity have been described based on the importance of completion of the maturation process. Redistribution of organelles within the cytoplasm has been described in the horse. Enlargement of the perivitelline space, and the rearrangement of membrane–bound vesicles and mitochondria gives the mature oocyte a polarized appearance (Grondahl et al, 1995). Moreover, the mitochondria relocation during in vitro maturation is influenced by hormones and energy substrates in the medium, and their appearance varies between stages of maturation and with oocyte quality. This has been associated with the level of ATP content in each individual oocyte, which is a predictive value of the oocyte's developmental potential (Stojkovic et al, 2001).

Glutathione (GSH) is a major non-protein sulphydryl in mammalian cells that serves as a reservoir for cysteine and plays an important role in protecting the cell from oxidative stress (De Matos et al, 2000). Glutathione content increases during oocyte maturation as the time of ovulation approaches, and glutathione concentrations are higher in oocytes matured in vivo than in vitro (Funahashi et al, 1994). It also participates in sperm decondensation in parallel with oocyte activation, and in the transformation of the fertilizing sperm head into the male pronucleus. In pigs, levels of glutathione have been associated with higher

blastocyst development; therefore, this could be used as a measurement of cytoplasmic maturity (Coy et al, 1999).

The organization of microtubules play an important role in chromosomal movement and cell division, and they have been described in the horse (Tremoleda et al, 2001).

Cortical granules are membrane-bound secretory granules located immediately beneath the plasma membrane in many vertebrate and invertebrate oocytes. Typically fertilization triggers fusion of cortical granule membranes with the plasma membrane, resulting in exocytosis of the contents of the granules. This results in the block to polyspermy and indicates that the cytoplasm is ready to process the sperm cell (Cherr et al, 1988). Therefore it has been extensively used as a measurement of cytoplasmic maturation (Prather et al, 1998). In the horse, patterns of cortical granule migration have been observed in immature and mature oocytes (Goudet et al, 97; Carneiro et al, 2002; Franz et al, 2002).

All the processes described above occur in synchrony in a maturing oocytes, and the analysis of a single aspect may not translate into perfect function of the other aspects (e.g. polyspermy in pigs). Moreover, evaluating these processes may be visually comforting, but the oocytes need to be killed to assess these parameters. The ultimate test for evaluating the readiness of the cytoplasm and nucleus of the oocyte is formation of embryos after fertilization. Unfortunately, this is difficult to achieve in vitro and expensive in vivo in the horse.

***Maturation medium***

This section of the review will provide an overview of oocyte maturation media. The experiments in this dissertation did not test hypotheses of effectiveness of specific maturation media components, but adequate maturation systems are essential for development of in vitro techniques. Oocytes from different species have different requirements for IVM and also for embryo development after fertilization (Kito et al, 1997). The maturation medium used for in vitro maturation of equine oocytes was based on studies in cattle, which used a complex medium supplemented with pyruvate and/or lactate. The most widely used is tissue culture medium 199 (TCM 199), which was designed for general cell culture (Morgan et al, 1950); usually it is enriched with a protein source, such as fetal bovine serum or bovine serum albumin, which acts as a surfactant to facilitate manipulation and to prevent zona hardening and gonadotropins. Due to the low overall maturation rates in horse oocytes in the first decades of equine oocyte experiments, several different components were added to the maturation medium, such as equine pituitary extract (Bezard et al, 1992), follicular fluid (Bogh et al, 2002), estrous mare serum (Willis et al, 1991; Zhang et al, 1989), or co-culture with thecal or granulosa cells (Okolski et al, 1993). The results of these studies were interesting, with maturation rates varying between 38 to 93% (Choi et al, 2002) and highest when co-culture systems were used. The great variability of undefined components in these culture conditions led to using chemically defined media for in vitro procedures. To understand the requirements for

development of immature oocytes through IVM, all products should be eliminated from culture conditions (Ali et al, 2002). Recently, more attention is being paid to fulfill oocyte requirements for cytoplasmic maturation in order to enhance developmental potential after fertilization. This concept is especially important for the horse oocytes.

In the experiments presented here, the maturation medium was a synthetic oviductal fluid (SOF) base medium, which was initially designed for embryo culture based on the composition of sheep oviductal fluid, and produced a high percentage of viable blastocysts from early cleavage stage embryos in cattle and sheep (Tervit et al, 1972). The benefits of addition of amino acids to embryo culture medium have been demonstrated in sheep embryos (Gardner et al, 1998). Among the effects of amino acid supplementation, is that it increases the pool size of endogenous amino acids and stimulates de novo protein synthesis (Takahashi et al, 1992). Ammonia produced by spontaneous degradation of amino acids (especially glutamine) played an important role in development of abnormal in vitro produced calves, due to the fetal oversize syndrome, induced during embryo culture (McEnvoy et al, 1997, 1999). Therefore, the addition of essential and non-essential amino acids in embryo culture medium and replacement every 48 h became routine.

Based on these results, a modified SOF medium with gonadotropins, growth factors and amino acids, was tested in equine oocytes for maturation, and resulted in excellent cumulus expansion and good maturation rates (Maclellan et al, 2000).

The addition of hormones, for example, progesterone, which was detected in increasing concentrations in follicular fluid (Grondahl et al, 1995; Schmidt et al, 2000) and growth factors, such as epidermal growth factor and insulin-like growth factor-I (Carneiro et al, 2001) has became routine for maturation systems for equine oocytes. Other factors also affect equine in vitro oocyte maturation: source of oocytes (slaughterhouse vs. aspirated follicles), time of the year, stage of the cycle, oocyte type (expanded or compact cumulus cells, FN or CC chromatin within the GV) (Hinrichs et al, 2000; Brück et al, 1996), collection method (scraping vs. slicing or aspirating) (Dell' Aquila et al, 2001), temperature and time of transport (for slaughterhouse ovaries) (Franz et al, 2001), time in maturation medium, (Hinrichs 1993; Del Campo et al, 1995) and concentrations of hormones and growth factors (Carneiro et al, 2001), among others.

Unfortunately, our understanding of the requirements for maturation of equine oocytes has not progressed to developing a defined maturation medium. The relationship between nuclear maturation rates and optimal development has not yet been determined.

## MATURATION PROMOTING FACTOR

Maturation promoting factor (MPF) was first described in Xenopus eggs when the contents of an egg were injected into an oocyte, inducing it to enter the M phase (Masui and Market, 1971). Later it was found in metaphase cells of all eukaryotes, implying that it contained evolutionary conserved components (Gautier et al, 1990).

### *MPF components*

MPF was characterized in Xenopus eggs (Lohka, 1988) and starfish as a dimer containing a protein kinase of 34 kd and a 45 kd phosphoprotein (Nurse, 1990). The 34 kd subunit was identified as an homologe of the cdc2 gene product of Schizossacharomyces pombae (Dumphy 1988; Gautier 1988). The 45 kd subunit was identified as a type B cyclin (Meijer, 1989; Labbe, 1989). Cyclins were isolated in starfish as a class of proteins that are destroyed at the end of M-phase (Evans, 1983). Cyclins A and B are correlated to M-phase activation while other classes of cyclins function at other times in the cell cycle (Parrish, 1992, Taieb et al, 1997). The association of both components of MPF resulting in pre-MPF, was first demonstrated in clam extracts (Draetta, 1989).

### *MPF activation*

Activation of MPF requires a complex series of reactions of phosphorylation and dephosphorylation on several sites (Nurse, 1990; Gautier et al, 1991). First, p34 cdc2 in an unphosphorylated form associates with cyclin; second, the (cell division cycle) cdc2

subunit of the complex becames phosphorylated on two threonines (T-14 and T-161) and one tyrosine (Tyr 15); third, dephosphorylation of the Tyr -15 and Thr-14 residues activates the complex (Gautier et al, 1989) while T-161 is maintained phosphorylated (Taieb et al, 1997). The structure of MPF and its activation are shown in figure 2.

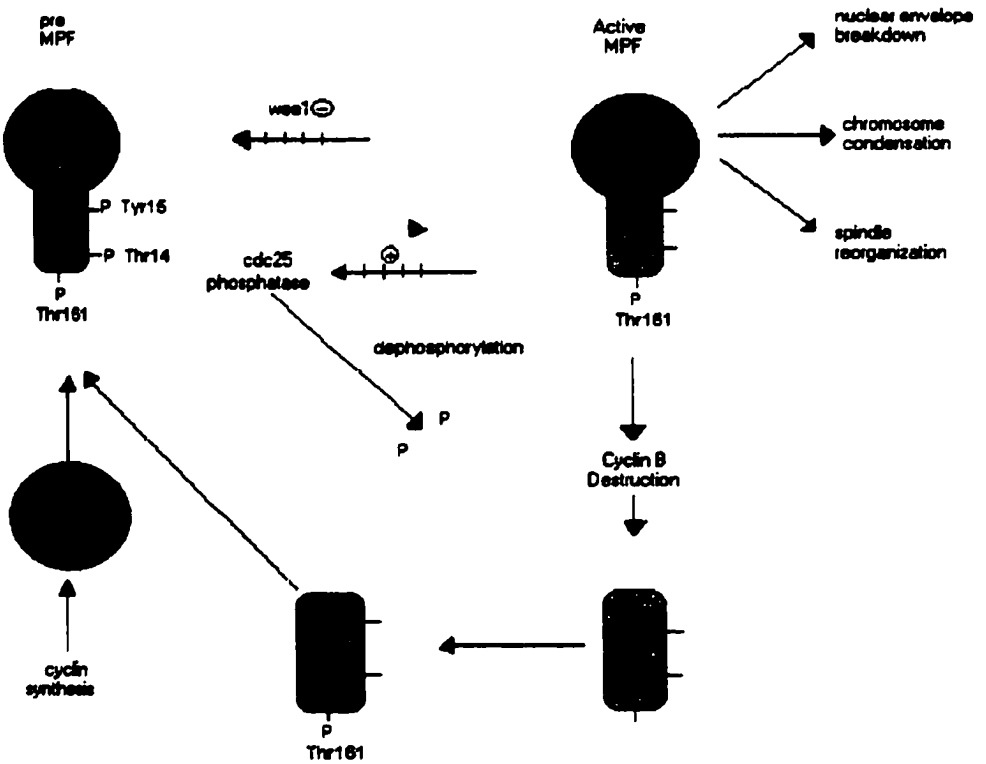



Figure 2 - Diagram of cdc2/cyclinB cycle. This diagram illustrates the relationship between cyclin, p34 cdc2, kinase activity and the cellular consequences of this activity. Newly synthesized cyclin B joins preexisting p34 cdc2 to form pre-MPF, which lacks H1 kinase activity until the cdc2 subunit is dephosphorylated. Cyclin is phosphorylated as the cdc2 becomes active and is subsequentely destroyed. This leads to loss of kinase activity, which cannot be restored until more cyclin has accumulated.

***This diagram was based on Gautier et al, 1990 and Parrish et al, 1992.***

Several gene products have been associated with MPF activation. The **cdc** 25 gene product is a protein phosphatase that contains both tyrosine and serine/threonine phosphatase activity (Gautier et al, 1991; Jessus et al, 1992). The cdc 25 protein also has an affinity for cyclin B and upon binding, the phosphatase activity is increased. This association (cyclin p34 cdc2 and cdc 25) peaks at the G2-M transition with activation of MPF (Jessus et al, 1992; Galaktinov et al, 1991). The phosphotyrosine is located at the ATP binding site, suggesting that part of the activation mechanism involves a change at this site, allowing the kinase to use ATP (Meijer et al, 1997).

***Effect of MPF on oocyte maturation***

During vertebrate meiotic maturation, MPF activity appears at the time of GVBD, decreases between the two meiotic divisions, and increases again at the second meiotic metaphase before it is stabilized by CSF (Taieb et al, 1997). Figure 3 demonstrates MPF oscillations through the meiosis process. Protein neo-synthesis is required in some mammals (sheep, goat, pig cow and horse) to activate MPF. However, in the mouse, accumulated cyclin B already is associated with cdc2 , resulting in a shorter latent period between the onset of maturation and GVBD (Motlik et al, 1990). The feedback mechanism that prevents the activation of p34 cdc2 until DNA synthesis is complete involves both inhibition of cdc 25 and activation of the kinase that phosphorylates Tyr 15. Tyr 15 phosphorylation is likely the product of the wee-1 and mik -1 genes, but this regulation is not direct because caffeine and okadaic acid can stimulate p34 cdc2 before

DNA synthesis is complete by threonine/serine kinase-phosphatase regulatory system upstream of the wee-1 kinase (Smythe et al, 1992).

In oocytes, active MPF induces chromosomal condensation, nuclear envelope breakdown and spindle formation (Lohka and Maller, 1985, Gautier et al, 1988; Wu et al, 1997). Phosphorylation of nuclear lamins by P34 cdc2 seem to be crucial for the solubilization of the lamin proteins and nuclear envelope breakdown (Parrish et al, 1992). Similarly, cdc2 phosphorylation has been associated with changes in the microtubular dynamics of the centrosome in Xenopus eggs (Verde et al, 1990)).

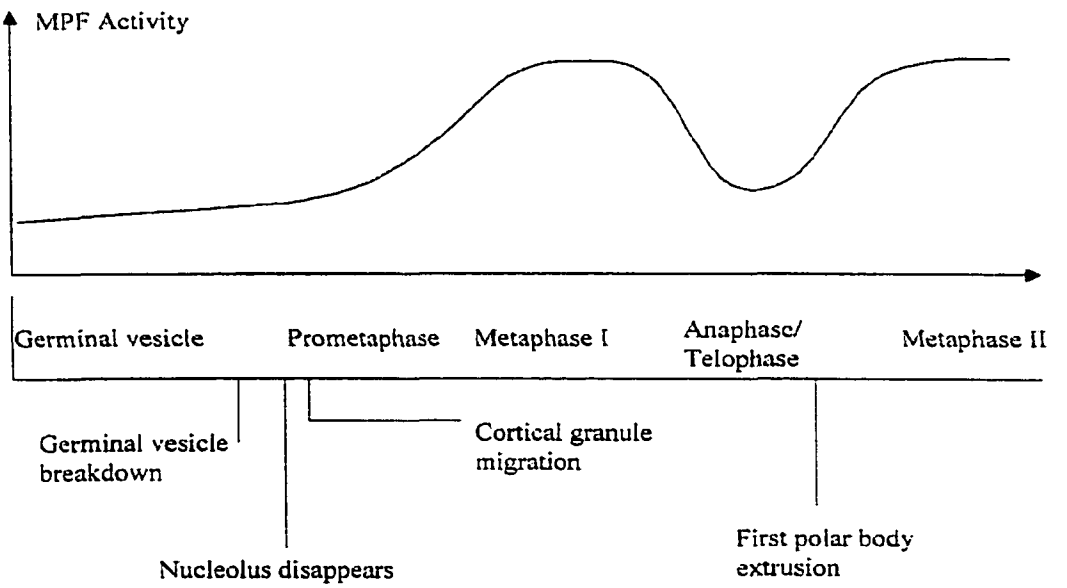



Figure 3- Levels of MPF during oocyte maturation. Shortly after GVBD, the nucleolus disappears and the cortical granules migrate to the sub-cortical area, due to the rearrangement of the microfilaments. Levels of MPF decrease to basal levels at anaphase and telophase, enough to maintain chromosomes condensed, and then increases again at MII.

***Histone Kinase activity in oocytes***

Fluctuations in histone kinase activity during the cell cycle were first reported in mitotic hamster cells (Lake and Salzman, 1972) and later in xenopus and starfish eggs (Karsenti et al 1987; Meijer and Podaven, 1988). The ability of MPF immunoprecipitated with cdc2 antibody to express H1 kinase activity was attributed to the p34 cdc2 component (Gautier et al, 1988). Anti-cyclin immunoprecipitates from clam embryos displayed histone kinase acitvity as a consequence of the association of cyclin B and p34 cdc2 (Draetta, 1989). Therefore, the histone H1 became a substrate commonly used in kinase assays to determine the activity of MPF in various cells including mammalian oocytes, as first described in the pig (Naito et al, 1991). In that study, time in culture, stage of maturation and levels of histone H1 kinase were correlated: at 4 h, all oocytes were in the GV stage, and the levels of histone kinase were low (16.7±5.8 fmol/h/oocyte) at 24 h; 39% and 29% of the oocytes were at first prometaphase and first metaphase. Oocytes in prometaphase after 24-28 h had kinase levels of 69 ± 16.3 fmol/h/oocyte and at 31 h the kinase levels detected were 95±11.7 fmol/h/oocyte. At 35 h most oocytes were in MI. Some oocytes were already in MI after 31 h with levels of 190±31.3 fmol/h/oocyte. Oocytes that were in anaphase I and telophase I during the same time period had low levels of histone kinase activity (6.1±19.9 fmol/h/oocyte). At 45 h, 85% of the oocytes were in MII and levels of histone kinase had risen again to the 184 ± 55.6 fmol/h/oocyte. Similarly, in cattle, low levels of H1 were detected for the first 8 h of maturation, which corresponds to oocytes in the GV stage. The initial peak occurred at 12h –14h (onset of MI), followed by an abrupt decline at 16-18 h (anaphase and telophase-I), increasing again at 20h (MII) and remaining at a plateau until fertilization

(Wu et al, 1997). This demonstrates that the fluctuation of MPF during maturation matches well with nuclear progression (figure 3). Its activity increases sharply at the G2 to M-phase transition, decreases at the anaphase and telophase stages, and then increases again at the metaphase of the second meiotic division. This has been demonstrated in mice (Hashimoto et al, 1988), rabbits (Jelinkova et al, 1994), goats (Dedieu et al, 1996), and cattle (Wu et al, 1997).

Media play an important role in the outcome of all in vitro processes; therefore, media that result in lower maturation rates, and lower pronuclei formation, result in lower levels of histone kinase (Naito et al, 1990). Despite the evidences that nuclear maturation is correlated to levels of MPF, experiments fusing two oocytes at different stages of maturation indicate that the cytoplasmic components modulate the changes during progression of meiosis (Fulka et al, 1985, Nurse et al, 1990). Key cell cycle components of MPF have been determined in growing pig oocytes, but they remain incapable of converting these components to active MPF until the growth phase is completed (Christmann et al, 1994). Similarly in equine oocytes, cyclin B and p34 cdc2 have been isolated in immature as well as mature oocytes, but were less evident in oocytes derived from small follicles (Goudet et al, 1998).

***Interaction of MPF and MAPK***

MPF activity has also been associated with mitogen activated protein kinase (MAPK). MAPK is a kinase that requires dual phosphorylation of its tyrosine and threonine residues to be activated and participates in the regulation of oocyte maturation (Cobb et al, 1991; Ruderman et al, 1993). Two isoforms of MAPK (ERK1-p44 and ERK

2- p42) have been demonstrated in mammalian oocytes including the pig (Inoe et al, 1995), cow (Fissore et al, 1996), and mouse (Verlhac et al, 1993). In bovine and equine oocytes, ERK 2 is the more abundant and is probably correlated to later, post GVBD processes (Fissore et al, 1996; Goudet et al, 1998). Acitvation of MAPK is the result of a complex cascade of kinases, starting with MAP kinase kinase (MAPKK), which is phosphorylated by MAP kinase kinase kinase (MAPKKK) (Gotoh et al, 1995). The target proteins of MAPK are several cytosolic and nuclear proteins, among them lamins and cytoskeletal proteins (Posada et al, 1993; Colledge et al, 1994). In Xenopus and bovine oocytes, the product of the proto-oncogene c-mos seems to activate this cascade of events (Posada et al, 1993; Fissore et al, 1996). Unlike MPF, levels of MAPK vary among species: in mouse oocytes, MAPK is activated after GVBD, and it seems to be responsible for reactivation of MPF between MI and MII (Colledge et al, 1994). In large domestic species such as the pig and the goat, MAPK is activated after GVBD (Inoe et al, 1995; Dedieu et al, 1996), while in bovine it appears at the same time as MPF. CSF is postulated to maintain elevated levels of MPF activity, and therefore has been correlated with meiotic II arrest (Moos et al, 1995) by inhibiting cyclin B degradation (Taieb et al, 1997). CSF has been characterized, and its components were all identified from the MAPK cascade (Sagata et al, 1997). Also, the c-mos component has been identified as a component of CSF: when injected in Xenopus eggs cultured with progesterone, the resumption of meiosis is blocked. CSF has also been correlated to spindle assembly and microtubular configuration, representing an important element on normal developmental potential (Gordo et al, 2001).

### *Inactivation of MPF after fertilization*

Shortly after fertilization, the transient increase in cytoplasmic free $Ca^{2+}$ results in inactivation of CSF and MPF (Sun et al, 1992). Upon fertilization the reduction of MPF activity (due to cyclin destruction) precedes the loss of CSF activity due to c-mos destruction (Moos et al, 1995). MPF must be inactivated for the cell to complete cytokinesis and return to interphase, and this is accomplished by maintaining p34 cdc2phosphorylated on Tyr -14 and Tyr-15 (Dumphy et al, 1994). MPF levels drop and remain low until the first mitotic division. This decrease in MPF is not associated with a decrease in cdc2 (which remains phosphorylated), but it has been clearly associated with a decrease in cyclin in bovine and pigs (Liu et al, 1999; Sun et al, 1992). Cyclin is a short half-life protein and accumulates again right before the first mitotic division (Nishijima et al, 1997). Shortly before the initiation of mitosis, cdc2 is dephosphorylated, resulting again in active MPF. Again, the accumulation of cyclins and the phosphorylation state of cdc2 are important in determining the entrance to mitosis. Moreover, the balance of phosphate seems to play an important role in the activation of MAPK. MAPK remains at high levels during incubation of sperm and eggs and drops around 10 h later, the time of pronuclei formation, when it becames dephosphorylated in ERK2 and inactivated, suggesting that it is associated with development of the pronuclear membrane and microtubular network (Liu et al, 1998).

It was proposed in the 1970's that the mechanism to maintain meiotic arrest was mediated by cAMP (Bilodeau et al, 1993), and that hypoxanthine maintains high levels of cAMP within the oocyte, preventing meiotic resumption (Downs et al, 1989). Inhibition of meiosis was first achieved to study the cell cycle with chemicals that interfere directly with cAMP activity such as phosphodiesterase inhibitors (IBMX), cAMP analogs (dbcAMP) and purines (hypoxanthine and adenosine) (Sirard et al, 1988; Sirard 1990). Adenylate cyclase sitmulators (forskolin) have been applied successfully in the cow (Bilodeau et al, 1993), rat (Racowsky, 1984) and mouse (Guoliang et al, 1994).

***Cyclin Dependent Kinases (cdk)***

Protein kinases are involved in almost all intracellular regulatory pathways, and cyclin dependent kinases play an essential role in the cell cycle; they are composed by a catalytic unit (cdks) and a regulatory subunit (cyclins) (Alberts et al, 1994).

Cdk proteins are regulated by (a) transcription/translation of their subunits, (b) complex formation, (c) post-translation modifications (phophorylations/ dephosphorylations), (d) interaction with various protein inhibitors, and (e) modifications of their cellular localization (Morgan et al, 1995; Pines et al, 1995). Human tumor development is associated with numerous alterations of cdk proteins and their regulators, which is encouraging the search for selective cdk inhibitors (Cordon-Cardo, 1995; Karp et al, 1995). The abundance of cdc2/cyclin B kinase in starfish oocytes led to the

identification of several compounds that specifically inhibit cdc2. The first was 6-dimethylaminopurine (6-DMAP) (Meijer et al 1988; Neant et al, 1988).

***Roscovitine***

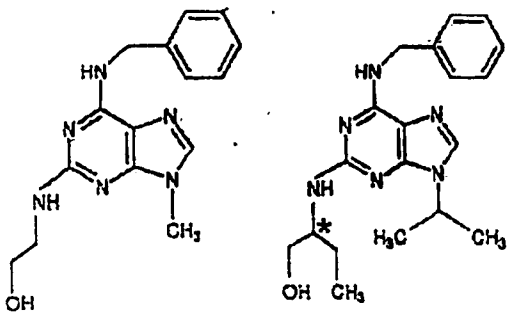
Roscovitine, [2-(ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine], is a recently characterized cyclin-dependent kinase inhibitor (cdk), which regulates the cell division cycle.

***The biochemistry of roscovitine***

Chemical studies of purine analogs resulted in the description of roscovitine (De Azevedo et al, 1997; Meijer et al 1997). By sucessive substitutions of purines, other cdc2 inhibitors have been described, such as: oloumucine, butyrolactone I, flavopiridol, suramin, and more recently roscovitine. Initially, when the inhibitory properties of these compounds on cyclin-dependent kinases and mitogen-activated (MAP) kinases were compared, olomoucine was the most effective inhibitor ($IC_{50}$: 7μM and 30μM, for cdc2/cyclin B and erk –1, respectively). Later, roscovitine was determined to be 10 fold more potent than olomoucine for MPF, $IC_{50}$: 0.65 μM. The chemical structures of roscovitine and oloumucine are compared in figure 4. The inhibition concentrations of roscovitine with olomoucine and 6-DMAP are shown in figure 5.

The (R)-stereoisomer of roscovitine binds strongly to cdck2, and is twice as potent in inhibiting cdc2/cyclin B than the (S) isomer. The mechanism of roscovitine action was determined by kinetic experiments with increasing roscovitine concentrations and varying ATP levels, demonstrating that roscovitine acts as a competitive inhibitor of

ATP, with the purine ring of roscovitine occupying approximately the same region as the purine ring of ATP (Meijer et al, 1997). Binding to cdck2 is characterized predominantly by hydrophobic and van der Wall's interactions with the same hydrophobic enzyme residues that form the pocket for the adenine base in the ATP complex structure.




Olomoucine Roscovitine

Fig.4- Chemical structure of roscovitine [2-(*R*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine] (102) and olomoucine [2-(2-hydroxyethylamino)-benzylamino-9-methylpurine] (51). An asterisk on roscovitine marks the asymmetric carbon.

Source: de Azevedo et al, 1997

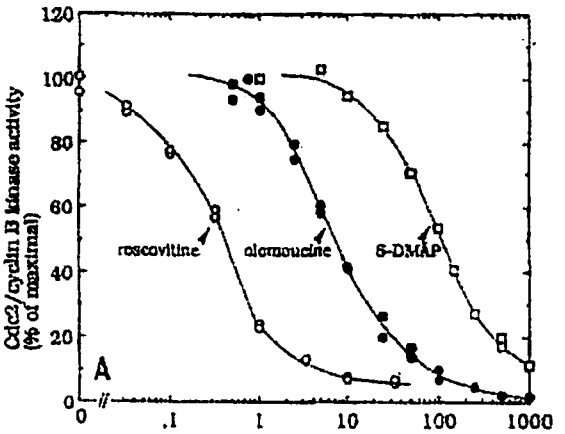



Fig.5- Roscovitine, olomoucine and DMAP dose response curves for p34 cdc 2/cyclin B.
Source: Meijer et al, 1997

***Cellular effects of roscovitine***

The effects of roscovitine in a few cellular models were described by Meijer et al, (1997). In starfish oocytes roscovitine inhibited nuclear envelope breakdown with an $IC_{50}$ of 5 μM, retarding (not inhibiting) tyrosine dephosphorylation of p34 cdc2. In mitotic sea urchin embryos, roscovitine arrested cells in late–prophase in a dose

dependent manner ($IC_{50}$: 10 μM), and its effect was completely reversible (with development into normal nuclei) even at 100 μM. In Xenopus egg-extracts, at concentrations of 0-5 μM, chromosomes appeared highly condensed and nuclear envelopes broke down. At 10μM and above, the interphase nuclei remained with partially decondensed chromatin and intact nuclear membranes, demonstrating that MPF was inhibited. In prophase arrested Xenopus eggs, a dose dependent inhibition of oocyte maturation was observed ($IC_{50}$: 25 μM).

***a) oocyte maturation inhibition in bovine oocytes***

The effects of roscovitine in mammalian cells was tested in nine tumor types and all displayed similar sensitivity to roscovitine, with an average $IC_{50}$ of 16 μM. The use of roscovitine in mammalian oocytes was first described in bovine oocytes (Mermillod et al, 2000). In that study, four concentrations were tested (12.5, 25, 50 and 100 μM) and a dose response was observed. The doses of 25, 50, and 100μM, maintained, respectively: 83±6, 89±3, and 82±9% of oocytes in germinal vesicle for 24 h, and the levels of histone kinase were maintained at the basal level for all concentrations. After release of inhibition, oocytes were incubated with 10 ng/ml of EGF and 84±5% of the oocytes reached metaphase II after 24 h of incubation in the permissive medium. The developmental potential of these oocytes was not affected, and blastocyst rates were similar to the control groups, with cleavage rates of 82±8 and 90±7%, for control and roscovitine, respectively. Another aspect shown in that study was that roscovitine was effective on maintaining oocytes in the GV stage, even in the presence of EGF (67± 11% and 78±11, for roscovitine + EGF or roscovitine alone, respectively).

In another study, roscovitine at 12.5 μM was only effective in inhibiting maturation of bovine oocytes in medium with BSA (3mg/ml) but not when 10% FCS was added; significantly higher inhibition of maturation was observed when doses ranged from 25 to 100μM (Calder et al, 2001). In the same study, oocytes were incubated with roscovitine for 24 h followed by 16 to 18 h in permissive medium resulting in 62.3% and 74.6% of oocytes in MII, respectively. This maturation rates (MII) were higher than the control groups at the same time points (16 h and 18 h in control medium, 35 and 50% MII; respectively), suggesting that blocking oocytes at the GV stage may accelerate the process of maturation.

The ability of bovine oocytes to cleave after maturation and fertilization was not compromised after treatment with 50 μM of roscovitine for 24 or 48 h. In fact, oocytes cultured with roscovitine for 24 h resulted in higher proportions of zygotes (3 days after fertilization) and blastocysts (9 days after fertilization) than oocytes cultured in roscovitine for 48 h or oocytes in the control group (81, 61, 52%, respectively for 3 day zygotes and 30, 17, 16%, respectively, for 9 day blastocysts) Mc Cann et al, 2001.

***b) Nuclear ultrastructure after inhibition of maturation***

In bovine oocytes, 4 to 8 h after the LH surge the nuclear envelope of the oocytes breaks down. It is initiated by a series of extensive convolutions of the nuclear envelope (NE) and chromatin condensation followed by the dissolution of the envelope into irregular vesicles and tubules resembling the endoplasmic reticulum (Desev, 1992). The nuclear structure of bovine oocytes after inhibition with 50 μM of roscovitine, 50 μM of butyrolactone-I or 2 mM 6-DMAP for 8 h was described by Faerge et al, 2001. The group of oocytes inhibited with roscovitine had a low degree of undulation of the

nuclear envelope when compared to the butyrolactone-I and 6- DMAP groups. Chromatin condensation was also evaluated, and roscovitine had less condensation, varying among different parts of the nucleus in individual oocytes, whereas the BL-I group had moderate condensation dispersed throughout the nucleus, with more condensed patterns surrounding but not in contact with the nucleoli. 6-DMAP groups had highly condensed chromatin in contact with the nucleolus. All changes were considered aberrant and varied among the arresting agents. Nevertheless, the significance of these findings is questionable since several reports showed evidence that roscovitine did not affect developmental potential of oocytes.

***c ) Roscovitine in porcine oocytes***

The use of roscovitine in pig oocytes has also been reported with success. The overall efficiency of in vitro produced embryos is lower in the pig than in sheep and cattle (Grupen et al, 2000) with high rates of polyspermy (25 to 57% - Bureau et al, 2000; up to 86%- Day et al, 2000), and blastocyst formation rarely exceeds 30% (Bureau et al, 2000 and Grupen et al, 2000). Therefore, the use of roscovitine to allow final differentiation in pig oocytes has been explored. Marchal et al, (2001) investigated the effectiveness of roscovitine in maintaining oocytes meiotic arrest for 22 or 44 h as well as the consequences for that inhibition. Unfortunately, the method of staining used (bisbenzimide) which is a specific chromatin stain that does not stain the nuclear membrane so the end point considered for the study was the resumption of meiosis, which is less specific than maintenance of oocytes in the germinal vesicle stage. In that study, the proportions of oocytes that had resumed meiosis after 22 h of inhibition with 12.5, 25 and 50 μM of roscovitine, were 25±13%; 8±5%, 13±10%, respectively,

demonstrating that 25 μM was sufficient to maintain a significant proportion of the oocytes in meiotic arrest. After additional 22 h in permissive medium the MII rates were: 69±8%, 72±7% and 59±3% for 12.5, 25, and 50 μM roscovitine, which was not different from the control group (44h): 61±8.5%. Furthermore, results of resumption of meiosis after inhibition were the same after 22 or 44 h in all three concentrations tested.

Controversially, Ju et al, (2001), inhibited pig oocytes for 44 h and reported that doses of roscovitine ranging from 10 to 50 μM were not effective in maintaining pig oocytes in GV, with large proportions of oocytes reaching MII (70-87%). Only when the doses were increased to 80 – 120 μM, 70 to 74% of the oocytes were maintained in GV.

When MPF activity was evaluated, roscovitine treated oocytes had levels similar to control oocytes in GV, with the 12.5 μM only partially inhibiting H1 kinase. However, after culture in permissive conditions, all three doses increased to the same range of histone kinase activity (Marchal et al, 2001). A decrease in MPF and MAPK kinase activity was also reported after the use of 50μM roscovitine in pigs by Krischek et al, (2000), when compared to control groups.

The developmental potential of pig oocytes after roscovitine was reported by Marchal et al, (2001). Blastocyst formation for oocytes inhibited for 22 h was lower than the control, but higher than that for oocytes cultured for 66h. However, the overall blastocyst rates in that study were low (6.1%, 10.7%, 3.5% and for roscovitine, control and 66h, respectively).

***Effects of combining two cdc2 inhibitors***

A combination of roscovitine and butyrolactone-1 was used by Ponderato et al, (2001); in bovine oocytes to evaluate the synergistic effect of these two agents that work in the same inhibitory pathway. The lowest effective combination (6.25 μM BL-1 and 12.5μM of roscovitine) was able to maintain 93±2.5% of oocytes in GV stage after 24 h of incubation. After release of inhibition followed by maturation in a permissive medium, 91.5±3% of oocytes were in MII. Cleavage and embryo development rates also did not differ between the treated and the control groups (68.5±4.4, 72.4±4.8, respectively for cleavage rates; 25.4±5.7 and 15.1±3.9, respectively for morulae; 25.7±8.1 and 16.2±4.5, respectively for blastocysts D+8). The kinetics of maturation were also evaluated in that study by fixing oocytes every two hours; inhibited oocytes reached MII faster than the control group (20 vs. 24 h, respectively) similarly to what has been indicated in the pig (Marchal et al, 2001; Krischek et al, 2000). This may indicate that a higher fertilization rate could be achieved if bovine oocytes were fertilized earlier, after incubation with roscovitine.

A recent study reported that the development of bovine fetuses is not affected by the use of a combination of 50 μM butyrolactone-I and 12.5 μM roscovitine (Ponderato et al, 2002) with 60% development after transfer, normal cardiac activity and normal extra-embryonic membranes and fetal size, at D+27. The GV rates reported for that study were 91.4± 1.4%; MII rates were not different between treated and control groups (89.5±2.0% and 88.3±5.5%, respectively) and neither were the embryo development rates and the survival after freezing and thawing (ranging between 18 and 31%). Although the researchers were looking for a synergistic effect, with resulting improvement of overall

development, oocytes treated with a combination of inhibitor agents were only similar, but not better than controls. If agents from different classes were tested, different results might be expected. Another important aspect of this study was that the overall proportions of end points (e.g. embryo development) were high, making it difficult to improve results further, under these in vitro conditions.

***Use of roscovitine in cloning***

For the near future, more dramatic effects of roscovitine might be seen in cloning experiments, where perfect synchronization of donor and recipient cell are crucial for further development. In fact, the use of 15 μM of roscovitine to synchronize bovine granulosa cells (donor cells) for nuclear transfer has been described, and resulted in greater numbers of cells in the G1-G0 phase than the serum starved (0.5% FCS) groups and the control groups (Gibbons et al, 2002). In that same study, roscovitine treated cells had more embryos at the blastocyst stage than the serum-starved reconstructed cells, and survivability of the calves produced was higher in the roscovitine treated groups. These results indicate that the use of roscovitine may impact the cloning industry in all species, due to dosage flexibility.

# IN VITRO FERTILIZATION OF EQUINE OOCYTES

Traditional in vitro fertilization, which is simply adding sperm to matured oocytes in vitro was described in cattle in 1982 (Brackett et al), followed by pigs, sheep and goats all in 1985 (Cheng et al, Hanada et al).

***First Reports in horses***

In 1989, Bezard et al, induced ovulation in mares with crude equine gonadotropins and collected oocytes from the follicles 34 h later. Mature oocytes were fertilized in vitro, and despite the cleavage rates observed in 6 of 20 oocytes after 20 to 70 h, early embryonic development was only normal in 2 of the embryos. Abnormalities were attributed to polyspermy since more than one sperm cell was found in each cleaved embryo. Nevertheless, the results suggested that in vivo matured oocytes could be fertilized by sperm previously capacitated with calcium ionophore A23187. Also in 1989, Zhang et al., collected equine oocytes from slaughterhouse ovaries, matured them in vitro from 24 or 30 h and then transferred to the oviduct of previously inseminated mare. Eight days later, embryos were collected. Fertilization had occurred in vivo, but it was an indication that in vitro matured oocytes could support fertilization. Interestingly, only oocytes that had expanded cumulus cells before maturation resulted in embryos.

In vitro maturation followed by in vitro fertilization of equine oocytes collected from slaughterhouse ovaries was described in 1990, with fertilization rates ranging from 15 to 33% (Del Campo et al, 1990 and Zhang et al, 1990, respectively). Results of these

two studies were complementary: pronuclei formation after 18 h was reported in 86% of the oocytes fertilized in the study of Del Campo et al, and 24% of the fertilized oocytes were cleaved 24 to 48 h after fertilization in Zhang et al. Interestingly, the maturation times for both studies were slightly different: 48 h IVM for Del Campo et al (1990), and 36 to 42 h for Zhang et al (1990).

Traditional IVF in horse had a breakthrough in 1991, with the report of the first foal born (Palmer et al, 1991). Several protocols with few replicates were described in that retrospective study: oocytes were collected from preovulatory follicles, and further incubated for various periods of time (1 to 12 h), capacitation was induced with heparin or A23187 ionophore resulting in an overall fertilization rate of 26% (n=60), and the cleavage rate was 18% with one live normal foal. Successful IVF in the horse was reported again in 1992 (Bezard et al), but again with the fertilization of an oocyte that had been matured in vivo plus transfer of the presumptive zygote to the oviduct.

***Manipulating the zona pellucida***

The slow improvements in in vitro embryo production (IVP) in the horse were blamed on improper capacitation of equine sperm, associated with few available oocytes for research (Squires, 1986), and several studies focused on sperm capacitation protocols (Blue et al, 1989). Therefore, micromanipulation of the zona pellucida, such as partial zona dissection and total (zona-free) or partial zona removal were used in equine oocytes after the human model (Malter et al, 1989), with encouraging fertilization results (12%, 86% and 52%, respectively).However these procedures were associated with a high level of polyspermy (13% and 29% for partial and total zona removal , Choi et al, 1994).

High cleavage rates (79%) followed by 45% morulae and 18% blastocysts, have been reported when the whole IVP system was improved by zona drilling with a strong acidic Tyrod's solution, capacitation of sperm with 1 μM calcium inophore (A23187) and co-culture of fertilized oocytes with bovine oviductal cells and mouse embryos (Li et al, 1995).

The thick zona pellucida surrounding the equine oocyte caused some concern about zona hardening similarly to a phenomena described in mouse and rat oocytes, where premature release of cortical granules increases the resistance of the zona to protease digestion (Downs et al, 1986). Therefore, studies involving agents that prevent zona hardening such as fetuin, which is abundantly found in fetal calf serum, were conducted without any increase of the fertilization rates. It was concluded that spontaneous zona pellucida hardening was unlikely to be the major cause of low fertilizability rates in the equine (Dell' Aquila et al, 1999; Boyazoglu et al, 2000).

***Insemination dose***

The concentration of sperm used for in vitro insemination was evaluated by Del Aquilla et al (1996), and results were similar for all three concentrations tested 13, 15 and 15% fertilization rates for 1, 5 and 10 million sperm cells/ml, respectively. In the same study, partial removal of the large cumulus cell mass resulted in a higher rate of pronuclei formation than intact oocytes (33 vs. 12%, respectively); after 72 h of culture, two embryos were formed: one in the 2 and one in the 4 cell stage. These were considered retarded due to the lack of support of cumulus cells. Grondahl et al, (1995), also reported two 8- to 16-cell embryos (2/60) that had developed in vitro after 72 h of

culture following in vitro maturation of oocytes (IVM) and IVF (17% to 26% fertilization rate), but no pregnancy resulted after transfer to a mare's oviduct. In addition to the difficulty in repeating the results reported previously, different criteria to evaluate fertilization are used by researchers, making available data hard to compare.

***Sperm capacitation protocols***

Capacitation enables sperm to undergo the acrosome reaction upon binding with the zona pellucida of the oocyte, representing a fundamental initial step to achieve fertilization (Bedford, 1983). It includes a decrease in the cholesterol/phospholipid ratio of the plasma membrane, alteration or removal of surface and integral proteins leading to increased permeability of the membrane, and influx of calcium (Grondahl et al, 1994). In natural fertilization, sperm are in the female's reproductive tract, with interaction among components of the female reproductive fluid, such as glysoaminoglycans (GAGs), albumin, and the cilia of the oviductal cells (Graham, 1996).

Heparin is a GAG that has been widely used to capacitate bovine, rabbit and human sperm in vitro (Parrish et al, 1988; Lenz et al, 1983; Roy et al, 1985), but has resulted in low penetration rates in the horse (Grondahl et al, 1995; Dell' Aquilla et al, 1996; Zhang et al, 1990). Moreover, heparin was only effective to induce capacitation in stallion sperm at $\geq$ 50μg/ml (Farlin et al, 1993). The combination of caffeine and dbcAMP, crucial for capacitating rhesus monkey spermatozoa in vitro, was not effective in horses (Zhang et al, 1990). Similarly, Blue et al. (1989) reported a lack of an effect in the ability of horse sperm to penetrate zona free hamster eggs when caffeine was added to ionophore (A 23187).

The few reports on successful equine sperm capacitation have included the use of calcium ionophore A23187, but fertilization and cleavage rates are still low ranging from 2 to 33% and 3 to 24%, respectively (Del Campo et al, 1990, Zhang et al, 1990, Grondahl et al 1995). Calcium ionophore bypasses the calcium dependent regulatory mechanisms by allowing a massive influx of $Ca^{+2}$ (Watson et al, 1992). An influx of $Ca^{+2}$ is required to initiate acrosome reaction, which is a determining factor for the release of enzymes and membrane alterations for sperm-oocyte interaction (Fraser et al, 1987). High intracellular $Ca^{+2}$ concentration could be toxic (Alm et al, 2001), especially after freezing and thawing, which already destabilizes the sperm cell membrane.

The duration of exposure to ionophores has varied between 1 to 30 minutes, and the concentration varied from 0.1 μM to 10μM. (Graham, 1996). Ionomycin another calcium inophore, at 100 nM in combination with 8 bromo c-AMP resulted in 53% penetration rates in partial zona removed oocytes when the capacitation medium was supplemented with 1 mg/ml PVA (Choi et al, 2000).

Other compounds used for capacitation of stallion sperm include lysophospholipids (LPS) (Blue et al, 1989) and short–chain phospholipids (phophatidyl-choline- PC12) (Graham et al, 1987; Padilla et al, 1991, Hochi et al, 1996). Blue et al, (1989) reported a slightly higher incidence in sperm penetration rates of zona free hamster oocytes with LPS (46%) than with PC 12 (33%). Oviductal epithelial secretions (Ellington et al 1993), progesterone (Meyers et al, 1993; Rathi et al, 2002) and various procedures, such as sperm separation through a Percoll gradient (Arns and Shepard 1994), have also been investigated to promote capacitation of equine sperm, alone or in association with chemicals mentioned above.

***Effectiveness of equine sperm capacitation***

To assess the effectiveness of capacitation treatment, the acrosome reaction has been evaluated by electron microscopy (Grondahl et al, 1994), fluorescence conjugated agglutinin (Farlin et al, 1993), monoclonal antibodies (Zhang et al, 1990), esterase release (Salazar, 1998), tetracyclin (Kholkute et al, 1992), and homologous and heterologous zona pellucida binding and penetration (Fazeli et al, 1993; Choi et al, 1994 Choi et al, 1999; Bracket et al, 1982; Landim-Alvarenga et al, 2001). Due to the various assay systems employed, results are difficult to compare. Moreover, some studies used fresh semen and others used frozen semen (Grondahl et al, 1994). Individual factors associated with each stallion and the almost infinite combinations of treatments make development of a simple and effective capacitation system for the horse very difficult. Recently, with advances in molecular biology, specific pathways related to acrosome reaction have been identified in equine sperm, such as tyrosine phosphorylation (Rathi et al, 2002; Pommer et al, 2002).

The ultimate assay would be successful in vitro fertilization of equine oocytes, with repeatable results, as in other domestic species. Taken together, there is a strong indication that the lack of repeatability in in vitro production in horses is more likely due to improper or incomplete in vitro maturation of oocytes than solely to sperm capacitation or penetration ability (Grondahl et al, 1995; Alm et al, 2001). Recently, the timing of first events after equine IVF has been reported (Torner et al, 2002), demonstrating that penetration rates increased with incubation time (14% at 2 h and 46% after 32h), but the overall motility of sperm decreased significantly to 3% after 32 h of

incubation. This suggests that the oocytes completed maturation while incubating with the sperm cells.

In conclusion, it appears equine sperm can be adequately capacitated, and can penetrate the equine oocyte, but the timing required for both gametes to proceed is out of synchrony. Moreover, we conclude that IVF is possible in the horse, but early embryonic development is compromised resulting in very few embryos. Therefore research should focus on methods to improve the developmental competence of the oocyte so that sperm can be properly processed after penetration.

## INTRACYTOPLASMIC SPERM INJECTION

In vitro fertilization can be achieved easily in most invertebrates and those non-mammalian vertebrates with natural extracorporeal fertilization, but in mammals fertilization in vitro requires an environment that resembles the female genital tract, which makes it more difficult to achieve.

### *Bypassing the fertilization process*

It is believed that most IVF failures in horses were associated with the inability of the sperm to penetrate the zona pellucida. Therefore, injection of a single sperm cell into a mature oocyte, termed intracytoplasmic sperm injection (ICSI), has been investigated. Hiramoto, (1962) injected motile sperm diluted in physiological solution into sea urchin eggs, but the eggs remained unchanged. This result was in contrast to the observation of Graham (1966). While studying mitosis in Xenopus laevis, he injected a sperm into an oocyte and found that pronuclei formed. The technique was then perfected, and normal feeding tadpoles were obtained after sperm injection of Xenopus eggs (Brun et al, 1974). In mammals, ICSI was reported by Uehara et al. (1976): "microsurgical injection" of human and hamster sperm, with and without the tail and acrosome in hamster oocytes resulted in normal pronuclei. In vitro embryonic development to the blastocyst stage after ICSI was only achieved several years later in the mouse (Thadani, 1980). Despite being considered a unique species to study reproduction due to the ease of culturing oocytes and embryos, the birth of live mice after ICSI was only reported in 1994 (Roknabidi et al, 1994).

In humans, microinjection of human sperm resulted in pronuclei formation in human oocytes in 1988 (Lanzendorf et al, 1988), and the first live birth was described by Palermo et al, 1992. The high efficacy of this technique in humans and its rapid improvement associated with pressure by infertile couples, makes it the method of choice for male factor infertility using assisted reproductive techniques (ART) in human fertility clinics (Kimura et al, 1995; Tesarik et al, 1999;).

***ICSI in the equine***

In large domestic mammals, embryonic development after ICSI, often including live offspring, has been described in rabbits (Hosoi et al, 1988), cows (Goto et al, 1990), sheep (Clarke, 1988), pigs and cats (reviewed in Horiuchi et al, 2002). In vitro development after ICSI and transfer of two equine embryos at the morula stage were described by Meintjes et al, 1995; a vesicle indicating pregnancy was detected at 12 days, but lost between days 14 and 16. The first foal born after ICSI was in 1996 (Squires et al, 1996), the result of sperm injection into a matured oocyte collected from a slaughterhouse ovary. Oocytes collected from preovulatory follicles after induction of ovulation and injected with either fertile or infertile stallion sperm, and transferred immediately to a surrogate mares oviduct resulted in 1/8 pregnancy and a live foal (Mc Kinnon et al, 1998). In the same study, a group of oocytes was cultured, and 8/15 oocytes resulted in two-cell embryos after 24 h, and after immediate transfer into a recipient's oviduct 3 pregnancies resulted with another live foal. When oocytes were collected from slaughterhouse ovaries and matured in vitro, no embryo was formed after transfer to mice or mares' oviduct (Grondahl et al, 1997), despite the high rate of pronuclei formation

(50%) in that study. Based on these results, there was a strong indication that as long as the oocyte was matured in vivo, and zygotes were transferred without delay into an oviduct, live offspring could be produced using ICSI in the horse. This would be an alternative for subfertile stallions and mares, but could only result in one pregnancy per cycle, and is associated with the low success rates to produce live foals (around 10%).

The multiple follicles formed in the first trimester of gestation in the mare, raise the prospect of maturing oocytes in vitro for ICSI, since two fillies were born after oocytes of pregnant mares were matured in vitro, injected with stallion sperm and cultured for 48 h. However, the final success rate of live born foals was still low, 2/21 cleaved embryos transferred (Cochran et al, 1998). Unfortunately, most mares in need of this ART (including ICSI) are not capable of maintaining pregnancies, making this idea unrealistic for subfertile mares.

Since the first reports on successful ICSI, fertilization rates (with evidence of 2 pronuclei + 2 polar bodies) have varied between 14-58% (see table I). Initial cleavage rates up to 85% have been published (Li et al, 2000) and 30% blastocyst rates have been reported (Galli et al, 2000; Li et al, 2001). However, few offspring have been produced. In pigs, cleavage rates after ICSI are up to 73%, but there is high incidence of parthenogenesis. In cattle, cleavage rates up to 80% for 2 cell embryos and 30% blastocysts are repeatable. In conclusion, blastocyst rates in equine can be similar to bovine, but the repeatability is low, suggesting lack of understanding on the process.

### *Oocyte Activation*

When the spermatozoon binds and penetrates the zona pellucida to fuse with the plasma membrane with natural fertilization, sperm factors are released, and the oocyte is activated (Yanagida et al, 1998). A 33 Kd protein (oscillin) residing in the equatorial segment of the acrosome has been associated with oocyte activation (Kim et al, 1998). This is now know to be a special variant of phospholipase-C (Trounson et al, 2002). The result is exocytosis of cortical granules, completion of meiosis II, pronucleus formation, initiation of DNA synthesis and first cleavage (Li et al, 2000).

Artificial activation has been a standard procedure in ICSI for all species utilizing a wide range of inducers: electrical, chemical or physical (Yanagida et al, 1999). Recently, a combination of ionomycin and DMAP in bovine and mice has been used effectively. In the equine, calcium ionophore A23187, ionomycin, ethanol, thimerosal and inositol 1,4,5 phosphate have been effective in activating oocytes (Kato et al, 1997, Guignot et al, 1998, Schmid et al, 2000, Li et al, 2000). It has also been demonstrated that manipulation of sperm improves fertilization rates in humans (Catt et al, 1995), and that isolated sperm heads can efficiently activate oocytes (Kim et al, 1998).

In the horse, chemical stimulation is not necessary to induce oocyte activation after ICSI, and it increases parthenogenetic divison (Li et al, 2000). Similarly, Choi et al (2001); reported no need for activation system in the horse. The good results in their study were attributed to the Piezzo apparatus. With this equipment, sperm are immobilized by quick application of vibration, which damages the sperm membrane releasing sperm substances to promote oocyte activation after ICSI. The piezo micropipette-driving unit is able to advance the pipette holder at very short distance and

at very high speed, and has been used in mice, humans, bovine and equine oocytes (Kimura et al, 1995; Yanagida et al, 1998; Katayose et al, 1999; Choi et al, 2002; respectively). This technique is superior to conventional ICSI since it results in higher survival after the procedure, higher developmental rates and higher pregnancy rates Yanagida et al, 1998). This has been attributed to a more effective penetration in the oolema, less trauma incited in the ooplasm of the oocyte, and deep insertion of the sperm cell into the ooplasm. Taken together, this approach is a superior alternative to conventional ICSI for equine oocytes, as reported by Choi et al, 2002.

Table 1 - Summary of ICSI results in the equine

| Author | PN | 2-4 cell | 5-16cell | Blastocyst | Activation | observations |
|---|---|---|---|---|---|---|
| Cho et al 1995 | 30% | - | - | - | - | |
| Meintjes et al 1996 | 14% | - | - | - | - | |
| Dell Aquila 1997 | 30% | 7.2% | 3.6% | - | - | |
| Grondahl 1997 | 50% | 16% | 8% | - | - | |
| Kato et al 1997 | 21% | 1 | 4 | - | A23187 (10μM) | |
| Guignot et al 1998 | 68% | 46% | 5% | - | A23187 (10μM) | |
| Dell' Aquila 1999 | 42% | - | - | - | - | Used fetuin |
| Guignot 2000 | 40% | | | | | |
| Li et al 2000 | 51-58% | - | 30-50% | 22% | A23187 (5μM)+ 1%ethanol | |
| Dell Aquila 2001 | 48% | 2% | - | - | - | |
| Li et al 2001 | - | 46-65% | 25-70% | 7-30% | Ionomycin (1μM) | |
| Choi et al, 2002 | 65% | 43% | 55% | - | | |
| Galli et al 2000 | - | 74% | - | 24% | Ionomycin (5μM) | Transferred to sheep oviducts |
| Mclellan et al 2000 | - | 21-30% | 3-7% | 4% | 0.01 mM iaC | |
| Lazzari et al 2002 | - | 8-79% | - | 42-47% | | Rates are for morula and blastocyst |

# CHAPTER III

## Maintenance and reversibility of meiotic arrest in equine oocytes after incubation for 24 or 32 hours with roscovitine

## INTRODUCTION

The concept of maintaining oocytes in the germinal vesicle stage (GV) in vitro has been discussed in the previous chapters. The low repeatability of most in vitro experiments with horse oocytes, leads to the investigation of this concept applied to horse oocytes. Roscovitine was chosen since it represents an effective chemical in maintaining oocytes from other mammalian species in the GV stage without affecting their potential to undergo normal maturation once the inhibition is ceased.

## MATERIAL AND METHODS

### Collection of oocytes

Equine ovaries were collected at a local abattoir in Brazil and rinsed in warm saline (35 to 37°C) upon collection. Follicles ranging from 5 to 22 mm in diameter were scraped with a bone curette into individual dishes and rinsed with TCM 199 buffered with 20 mM MOPS at 35 to 37°C. The total time from slaughter to incubation of oocytes in culture medium was 1 to 5 h. Dishes were searched, and oocytes were classified into expanded and compact groups based on the surrounding cumulus cells and placed in TCM 199 with MOPS at 37°C. Every 45 to 60 min, new batches of classified oocytes were transferred to 5 ml Falcon tubes (Catalog # 352058, polystyrene) containing 1 ml of

medium (maturation medium or roscovitine medium, explained below) covered with 2 ml of light paraffin oil, both previously equilibrated in 5% $CO_2$ in air. The Falcon tubes were transported to the laboratory in a portable incubator (model 19180/2000, Verona -WI)) regulated at 38.2 °C.

## Oocyte maturation

Upon arrival at the laboratory, oocytes were removed from the transport tubes and incubated in 500 μl of the same medium in four well dishes (Nunclon, Demark) at 38.2°C in 5% $CO_2$ in air. All components were purchased from Sigma unless stated otherwise. Base medium was EMM1 (Maclellan et al, 2001) with 8% BSA (Serologycals® Kankakee, IL), 11 mM myoinositol (Sigma), 10 μg/ml streptomycin sulfate and 1000 IU/ml penicillin G. The control (maturation) medium was the base medium with 15 ng/ml ovine FSH (NIDDK-oFSH-20), 1μg/ml bovine LH (AFP-11743B) 1μg/ml $E_2$, 500 ng/ml progesterone, 100 ng/ml EGF, 10 ng/ml IGF-1 and 10% fetal bovine serum (Gibco).

In experiment I, cumulus oocytes complexes were incubated for 30 h. Roscovitine was diluted in DMSO at 28.2 mM and added to the base media at three concentrations: R1 – 22 μM, R2 – 66 μM, R3 – 200 μM. This experiment was repeated 5 times.

In experiment III, cumulus oocytes complexes were incubated for 24 h (R 24) or 32 h (R 32) with 66 μM of roscovitine followed by 24 h incubation in maturation media. This experiment was repeated three times. A control group was incubated in maturation medium for 32 h.

### Oocyte evaluation

After in vitro maturation all cumulus oocyte complexes were placed in warm Dulbecco's modified phosphate-buffered saline with 300 IU/ml hyaluronidase type IV-S to dissociate cumulus cells. A fine bore glass pipette was used to further strip attached cumulus cells. Oocytes were fixed in ethanol and glacial acetic acid (3:1) solution for 48 h before staining with 1% orcein in 99% acetic-acid. Fixed oocytes were then examined under a microscope to assess chromosomal configuration (Fulka et al, 1981). Oocytes were considered to be in M II if a metaphase plate and a polar body could be identified in the same oocyte.

## Histone activity:

### Oocyte preparation

Oocytes for experiment 3 were all collected during 4 trips to the slaughterhouse and individual oocytes at different stages were denuded of cumulus cells, washed 3 times in a buffer solution (80 mM ß-glycerophosphate, 2 mM PMSF 15mM 3-( n-morpholino) propanesulfonic acid (MOPS) pH 7.2, 15mM $MgCl_2$ , 10mM EGTA, 20µg/ml leupeptin, 10 µg/ml c-AMP-dependent protein kinase inhibitor peptide and placed in 0.3 ml Eppendorf tubes with 10 µl of buffer solution. Oocytes were stored frozen at -80°C until the assay was performed. Before the assay, oocytes were frozen and thawed once to break the membrane and centrifuged at 8000 g for 1 min.

Kinase assay:

Kinase reactions were initiated with the addition of 10 µl of substrate buffer containing: 2 mg/ml histone H1 (Type III s), 2 mM dithiothreiol (DTT) and 10 µCi [γ32 P ] ATP. The reaction were carried out for 45 min at 37 °C; 1 mg/ml deoxycholate was added to each individual tube, and tubes were placed in ice for 30 min. Next, 100 µl of TCA (20%) was added to each tube, and the samples were centrifuged at 14,000 g for 10 min at 6 °C. The supernatant was removed and discarded. This washing procedure was repeated identically a second time. After a second wash, 10 µl of NaOH (1N) and 15 µl of double–strength SDS sample buffer was added to each tube. The tubes were boiled for 3 min and loaded on an SDS PAGE gel. Each lane was loaded with one single oocyte preparation (see below). The gel was run at 200 V and transferred to blot paper, dried and exposed to film for 24 h at - 80°C. After developing the film it was scanned into a computer file and quantitative analysis of the bands of interest was performed using the ImageQuant® computer program.

For the compact and expanded groups of oocytes, one lane was loaded with granulosa cells, and one lane was loaded with one oocyte matured for 48 h (positive controls). A lane was loaded with one oocyte at 0 h (before maturation), and one lane was loaded with all ingredients without oocytes (negative controls). Three lanes were loaded with oocytes matured for 16 h, and 3 lanes were loaded with oocytes matured for 30 h (maturation lanes). Four lanes were loaded with oocytes incubated for 24 h in roscovitine and 24 h in maturation medium, and 4 lanes were loaded with oocytes incubated for 32 h in roscovitine and 24 h in maturation medium (treatment lanes). The last lane was loaded

with Rainbow® (molecular weight standard). The gels were repeated 3 times for each oocyte type (expanded and compact).

### Statistical Analysis

Data were analyzed using a multi-factorial ANOVA with arcsin transformation and Tukey's test. Stages of meiosis other than GV (experiment I) were analyzed with chi-square. Gels were analyzed by one-way ANOVA. The interaction between oocyte type (expanded or compact) was evaluated by multi-factorial ANOVA.

## RESULTS

**Experiment I: Dose response to roscovitine in maintenance of meiosis.**

From 80 ovaries, 334 follicles were scraped, and 198 oocytes were collected; 26 oocytes were degenerated and discarded, and 8 were lost during transportation. Therefore, 164 oocytes were used for this study. Results are summarized in Table 2. From the 36 oocytes in the control group, 50% and 69% were in Metaphase II, for expanded and compact groups, respectively. For the cumulus cell complexes incubated with roscovitine, 7% to 73% were maintained in the germinal vesicle stage (GV). $R_2$ was more effective (P=0.03) than $R_1$ but equal to $R_3$ in maintaining the oocytes at the GV stage. Compact COC, were more efficiently maintained in GV than expanded groups for all treatments ($R_1$, $R_2$, $R_3$) (P=0.08). Figure 5 shows an oocyte in the GV stage. The proportions of oocytes in other stages of maturation are summarized in Table 3. The GVBD stage was defined as condensed chromatin without a visible nuclear membrane surrounding it. There were no significant differences between expanded and compact for

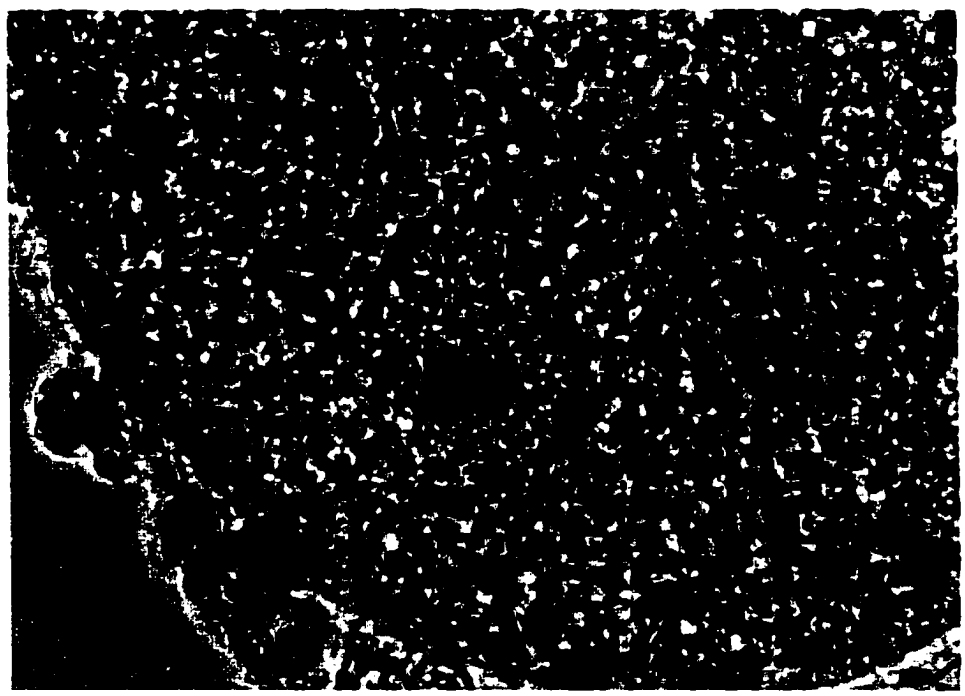
the responses in Table 3. However, a dose response for expanded and compact oocytes can be observed when MI rates are considered.

Table 2- Dose response of roscovitine in maintaining GV in equine oocytes

| | $R_1$ (22μm) (% GV±SE) | $R_2$ (66μm) (% GV±SE) | $R_3$ (200μm) (% GV±SE) |
|---|---|---|---|
| Exp | 1/15 (7±5)[a] | 8/21 (38±17)[b] | 15/27 (55±17)[b] |
| Com | 10/22 (45±13)[a,b] | 17/23 (73±11)[b] | 13/20 (65±14)[b] |

[a,b] Values within rows with different superscripts differ (P<0.05).

Figure 6- Equine oocyte (expanded category) in the germinal vesicle stage (arrow) after incubation with 66 μm of roscovitine for 24 h.



Stained with aceto-orcein
(magnification: 1000 X)

Table 3- Stages of maturation (other than GV) in equine oocytes incubated with roscovitine for 24h.

| Stage of meiosis | Oocyte type | $R_1$ (22μm) (%) | $R_2$ (66μm) (%) | $R_3$ (200μm) (%) |
|---|---|---|---|---|
| GVBD | Exp | (2/15) 13[a] | (2/21) 9[a] | (3/27) 11[a] |
| | Comp | (3/22) 14[a,b] | (0/23) 0[a] | (5/20) 23[b] |
| MI | Exp | (7/15) 46[a] | (8/21) 38[a,b] | (4/27) 15[b] |
| | Comp | (7/22) 32[a] | (4/23) 17[a,b] | (1/20) 5[b] |
| MII | Exp | (0/15) 0 | (1/22)4 | (0/27) 0 |
| | Comp | (0/22) 0 | (0/23) 0 | (0/23) 0 |

[a,b] Values within rows with different superscripts differ ($P< 0.05$).

**Experiment II: Reversibility of maturation block by roscovitine**

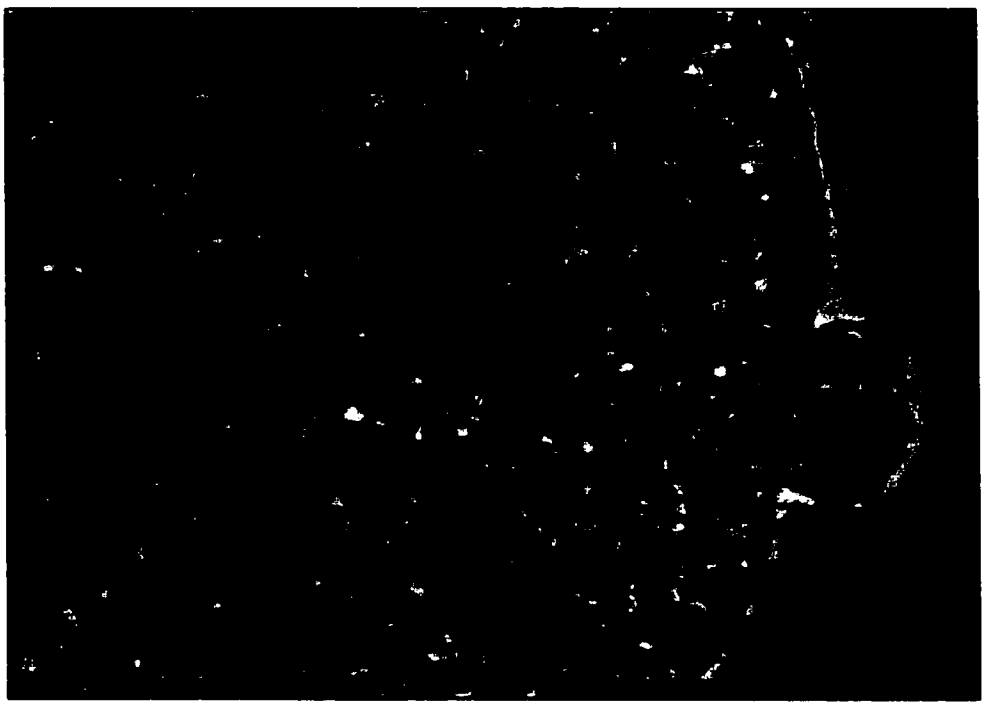
From the 73 ovaries used for experiment II, 313 follicles were scraped, and 174 oocytes were collected; 22 oocytes appeared degenerated, and therefore were discarded There was no significant difference ($P>0.05$) between the percentage of oocytes that reached MII among both treatments groups and the control group for either expanded or compact COC. Figure 6 shows a oocyte in MII. Percentages of oocytes reaching metaphase II ranged form 63 to 78% (Table 4).

Table 4- Nuclear maturation rates (MII) after inhibition with 66 μM of roscovitine

| | 24h $R_2$ + 24 h culture* (% MII±SE) | 32h $R_2$ + 24 h culture* (% MII±SE) | 32 h of culture* (% MII±SE) |
|---|---|---|---|
| Exp | 17/23 (73± 0.12)[a] | 14/19 (73±0.07)[a] | 18/23 (78± 0.04)[a] |
| Com | 22/32 (68±0.01)[a] | 21/33 (63± 0.01)[a] | 18/24 (75± 0.07)[a] |

[a] No significant differences ($P>0.1$).
*culture EMM1 (Maclellan et al, 2000)

**Figure 7-** Equine oocyte (compact category) in metaphase II after incubation with 66 μm of roscovitine for 32 h and incubation in maturation medium for 24 h. The dotted arrow indicates the metaphse plate and the solid arrow the first polar body.



Stained with aceto-orcein
(magnification: 1000 X)

**Experiment III:**

The effects of roscovitine on MPF activity after release of inhibition were evaluated by the histone H1 kinase assay. A representative gel is shown in Figure 5 and 6 indicating labeling of a 32 kd band. H1 activity was present in the 16, 30 and 48 h control treatments and also in the 24 h roscovitine + 24 h culture and the 32 h roscovitine + 24 h culture in both in compact and expanded categories.

In the compact group, there was no difference in qualitative analysis in the bands obtained for 16, 30 and 48 h. The levels of histone kinase activity (MPF) for 24 h roscovitine + 24 h culture and 32 h roscovitine + 24 h culture was not different from the 48 h control but different for the 16 and 30 h culture ($P<0.05$) (Table 5). The 0 h lane could not be accessed because it was next to the granulosa cell lane, which was very radioactive and interfered with the reading of the 0 h lane.

In the expanded category of oocytes, there was no difference between the 16, 30 and 48 h controls and the roscovitine inhibited groups after release of inhibition ($P>0.05$) (Table 5). Averaged over all treatments, means of 485,000 (±51,000) and 462,000 (±52,000) pixles were calculated for expanded and compact COCs. There was no interaction between cumulus type and treatment ($P>0.05$).

Table 5– Means of MPF activity, calculated by the area of each corresponding band using Image Quant® (number of Pixels, $10^3$).

| Treatment | Compact COC (± SE) | Expanded COC (± SE) |
|---|---|---|
| 16 h | 680 (± 66) [a] | 590 (± 95) [a] |
| 30 h | 599 (±33) [a] | 499 (±81) [a] |
| 48 h | 547 (±33) [a,b] | 516 (±143) [a] |
| 24R+ 24 h | 273 (±100) [b] | 388 (±148) [a] |
| 32R+ 24 h | 326 (±109) [b] | 317 (±113) [a] |

[a,b] Values within columns with different superscripts differ ($P<0.05$).

# DISCUSSION

This study demonstrated that roscovitine, a specific inhibitor of cyclin dependent kinases, blocks equine oocytes at the GV stage for up to 32 h without affecting their ability to resume meiosis after being transferred to a permissive medium. Retarding the spontaneous nuclear maturation of oocytes that occurs in vitro might reproduce events that occur in vivo during late folliculogenesis. This would be particularly important in mammalian species that require neo-synthesis of protein to resume meiosis; and could increase the oocytes' ability to develop into an embryo after fertilization (Ponderato et al, 2001). Recently, it has been demonstrated in bovine oocytes that developmental competence increases during meiotic arrest induced by butyrolactone-I, another kinase inhibitor (Hashimoto et al, 2002).

In experiment I, a dose response to roscovitine was observed from 22 ($R_1$) to 66 μM ($R_2$) without further increment at a higher dose of 200μM ($R_3$) in compact and expanded oocytes groups. Moreover, an evident dose response was determined when analyzing the proportions of oocytes in metaphase I in the compact category: 31, 17 and 5 % of COC were in MI, for $R_1$, $R_2$ and $R_3$, respectively. A similar dose response was observed in the expanded category with MI rates of 46, 38 and 15 % $R_1$, $R_2$ and $R_3$, respectively. In Xenopus eggs, roscovitine slows down tyrosine dephosphorylation of p34 cdc2 in a dose dependent manner with an IC 50 of 25μM (Meijer et al, 1997). In mammalian species, the use of roscovitine was first described in bovine oocytes. It was effective in maintaining GV for 24 h, without compromising further developmental

potential in concentrations of 12.5, 25, 50 and 100 μM. The maximum level of inhibition (83±6% in GV) was obtained at 25 μM after 24 h (Mermillod et al, 2000). Ju et al, (2001); observed the highest inhibitory effect on maturation in pig oocytes when concentrations of 80 to 120 μM of roscovitine (70-74%) was used for 44 h.

In this study, there were 4 oocytes in MII after 24 h of incubation with roscovitine in the expanded group. We assume that these oocytes were probably in metaphase II when they were collected. In the horse, oocytes with expanded cumulus cells need less time in culture and have higher potential to develop in vitro. This suggests that a higher dose of roscovitine might be required to maintain oocytes in GV stage in expanded oocytes. Despite the high reported specificity of roscovitine to cdc2/cyclin B, an effect on MAPK levels was demonstrated in pig oocytes (Krischek and Meineke 2000), suggesting that higher doses of roscovitine may affect the MPF as well as MAPK activity. In the horse, preovulatory competent oocytes (therefore expanded) had evidence of phosphorylated MAPK, while in immature oocytes MAPK was not phosphorylated (Goudet et al, 1997). These results might suggest that MAPK activation occurred without MPF activation and GVBD. Similarly, Marchal et al, (2001), reported that a lower dose of roscovitine is less effective in pig oocytes due to residual MAPK and MPF activities.

When horse oocytes were inhibited for 16 h with cycloheximide, more expanded that compact oocytes were in MI (12.1 % vs. 2.9 %) and MII (51.5 % vs. 17.6 %), demonstrating that expanded COC need less time for protein synthesis (Alm et al, 1996). This suggests that some proteins had already been accumulated in oocytes from the expanded category, which corresponds to the oocytes more advanced in the maturation process.

In the bovine, 12.5 μM roscovitine is effective in inhibiting MPF activity but it is poorly effective in inhibiting MAPK, allowing the oocytes to resume meiosis, slowly reaching metaphase I (Mermillod et al, 2000). Great variability of stage of maturation at collection in expanded COC has been reported in horse oocytes at time of collection (Del Campo et al, 1992; Hinrichs et al, 1993; Willis et al, 1991; Torner and Alm, 1995). When higher doses of roscovitine were used in bovine oocytes, proportionally lower MPF activity was reported without compromising further development (Mermillod et al, 2000).

The nuclear ultra structure of bovine oocytes has been evaluated after inhibition of meiosis with DMAP, butyrolactone-I and roscovitine; the roscovitine-treated oocytes had only a small undulation and moderate chromatin condensation (Faerge et al, 2001). Similarly, a more distinct inner cell mass of bovine embryos produced after inhibition with roscovitine was described (Ponderato et al, 2001).

In our study, the morphology of the chromatin within the GV was condensed in all roscovitine-treated oocytes, regardless of their initial cumulus classification (Figure 4). Similarly, Kubelka et al, (2000), reported 86% of bovine oocytes maintained in GV with butyrolactone-I for 24 h had condensed chromatin. The initial chromatin configuration has been extensively studied in the horse (Hinrichs et al, 1993; Gable et al, 2001). Equine oocytes with diffuse chromatin within the GV are less competent than those with condensed chromatin (Hinrichs et al, 2000). In bovine oocytes, initial chromatin configurations were filamentous at the time of collection in a study by Tatemoto et al, (1994). This suggests that the process of chromosome condensation requires phosphorylation that can be affected by the use of kinase inhibitors. Taken together, this indicates that roscovitine modulates the meiosis process, which could favor

the synchronization of meiosis in vitro. The importance of improved synchronization of meiosis would benefit pig and horse oocytes obtained at slaughterhouses, which are heterogeneous in morphology and competence, leading to great variability of results (Funahashi et al, 1997; Marchal et al, 2001).

Roscovitine was evaluated in several types of cell lines, and when exposed to 120 μM some of them died rapidly (Meijer et al, 1997). Similarly, when butyrolactone-I was used at 200 μM in bovine oocytes, partial degeneration of the GV was observed (Kubelka et al, 2000). Results of experiment I demonstrated that 66 μM of roscovitine was sufficient to maintain GV; therefore this concentration was chosen to avoid higher doses that could be detrimental and affect reversibility. Also, in experiment II, we wanted to determine if longer inhibition would affect the final maturation rates. In this study, there was no difference between the control and the roscovitine treatment groups after 24 or 32 h of inhibition. Maturation rates of compact oocytes exposed to roscovitine were slightly, but non significantly lower than the control. The oocytes with compact cumulus that were incubated for 24 h after inhibition with roscovitine, were substantially similar to the groups matured for 32 h. Previous studies indicate that equine oocytes with compact surrounding cumulus cells need around 30 h to mature while expanded oocytes need only 24 h (Hinrichs et al, 1993).

Marchal et al (2001), observed no difference in reversibility after 22 or 44 h of inhibition with 25 or 50 μM of roscovitine in pig oocytes, but lower developmental potential of oocytes cultured for 66 h than 44 h. Nevertheless, developmental competence was higher in oocytes inhibited for 22 h and cultured for additional 44 than

those cultured for 66 without inhibition. This suggests that roscovitine can slow down the ageing process of oocytes exposed to long culture periods.

The permissive medium used in the current study (EMM1) contains hormones and growth factors, which stimulate oocyte maturation (Maclellan et al, 2001). Therefore, we anticipated that resumption of the maturation processes would occur faster after inhibition. Ponderato et al, (2001) demonstrated that some meiotic progression occurs during inhibition and that oocytes treated with a combination of 6.25 μM butyrolactone-I and 12.5 μM roscovitine were effectively maintained in GV for 24 h and resumed meiosis faster after being removed from the inhibiting medium. Similarly, Tatemoto et al, (1997) demonstrated that the sequence of GVBD occurred faster in bovine oocytes exposed to cycloheximide.

In experiment III, the level of MPF activity was evaluated after inhibition with roscovitine. Some variation between the lanes was observed and could be attributed to the somewhat heterogeneous population of oocytes, and also to the technique used. The modified technique used only one oocyte per lane, and the double centrifugation (with TCA) followed by aspiration of the supernatant could have accidentally removed some of the oocyte extract, since no visualization of the minuscule pellet was possible. Nevertheless, the results are in agreement with previous studies in the horse and other large animals where the bands of a 32 kd protein are evident in most of the lanes containing mature cells. In this study, the controls of 16 and 30 h in EMM1 were designed to evaluate oocytes in MI and MII, respectively. MPF levels increase at MI, decreased between MI and MII and raise again at MII in mouse, pig, bovine and goat oocytes (Naito et al, 1991, Wu et al 1997, Dedieu et al, 1996). At the germinal vesicle

stage minimal MPF activity has been reported in all these species. Controversially, in the horse, it has been demonstrated that immature control oocytes had low levels of MPF (32 kd band) but no evidence of a 44 kd band (Goudet et al, 1998), similar to the results shown in this study. The bands present in the lanes corresponding to the treatments of 24 h roscovitine + 24 h EMM1 and 32 h roscovitine + 24 h EMM1, were similar to the 16h, 30 h and 48 h controls, suggesting that the inhibition was reversible, and that oocytes fully recover after 24 h inhibition without compromising cytoplasmic activity. In the compact category, lower activity was detected in control oocytes at 16 and 30 h, suggesting that oocytes in the compact category had not accumulated some of the key elements for MPF formation. In contrast, in the expanded oocytes category, there was no difference between the 16, 30, 48 h controls and the treatment groups. Possibly oocytes in this category are already committed to mature, as discussed previously. Similar results were demonstrated in the pig (Marchal et al, 2001). On Experiment II, longer inhibition did not affect the subsequent ability of the oocytes to reverse the block and mature to MII. Visually, the most evident band, after the positive control of granulosa cells, was the 48 h control, which could be attributed to the fact that MPF is accumulated during maturation.

The less evident bands for the treatment groups, the key elements are still present and therefore might not decrease the ability to form normal embryos after fertilization. Emphasizing this hypothesis, Naito et al (1992); demonstrated differences in H1 kinase activities in pig oocytes subjected to two different media with no effect on the final maturation rates to MII after 48 h. Experiments focusing on the ideal time in culture and histone kinase levels could help refine this theory. The technique for measuring histone

kinase activity used in this study was an adaptation to evaluate individual oocytes, and it is an important tool for further molecular studies in the equine.

In conclusion, roscovitine efficiently and reversibly prevented resumption of meiosis in equine oocytes. This treatment can be used as a tool to manipulate the cell cycle and therefore the process of maturation, which could be useful for cloning projects. Moreover, it might provide a more homogeneous population of oocytes for studies on the kinetics of meiosis. The objective of using roscovitine was to increase the synchrony between the cytoplasm and the nucleus of the oocytes to imitate in vivo maturation. Other measures of cytoplasmic activity should be pursued to determine if there would be a clear advantage to use roscovitine in equine in vitro maturation. Ultimately, the ideal test to assess the cytoplasm would be to fertilize the oocytes after treatment with roscovitine and evaluate formation of pronuclei and cleavage, and then transfer the embryos to determine if normal pregnancies and offspring result.

**Figure 8-**

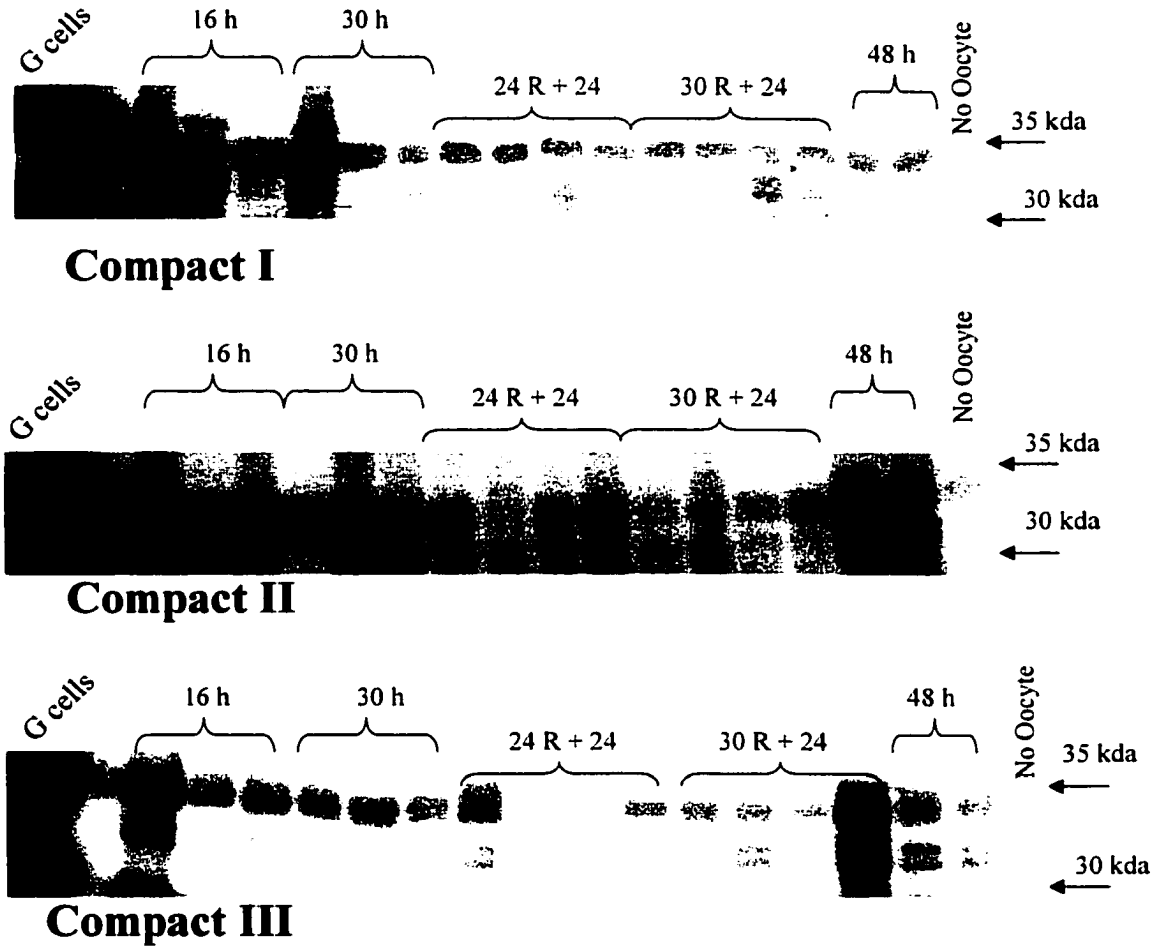
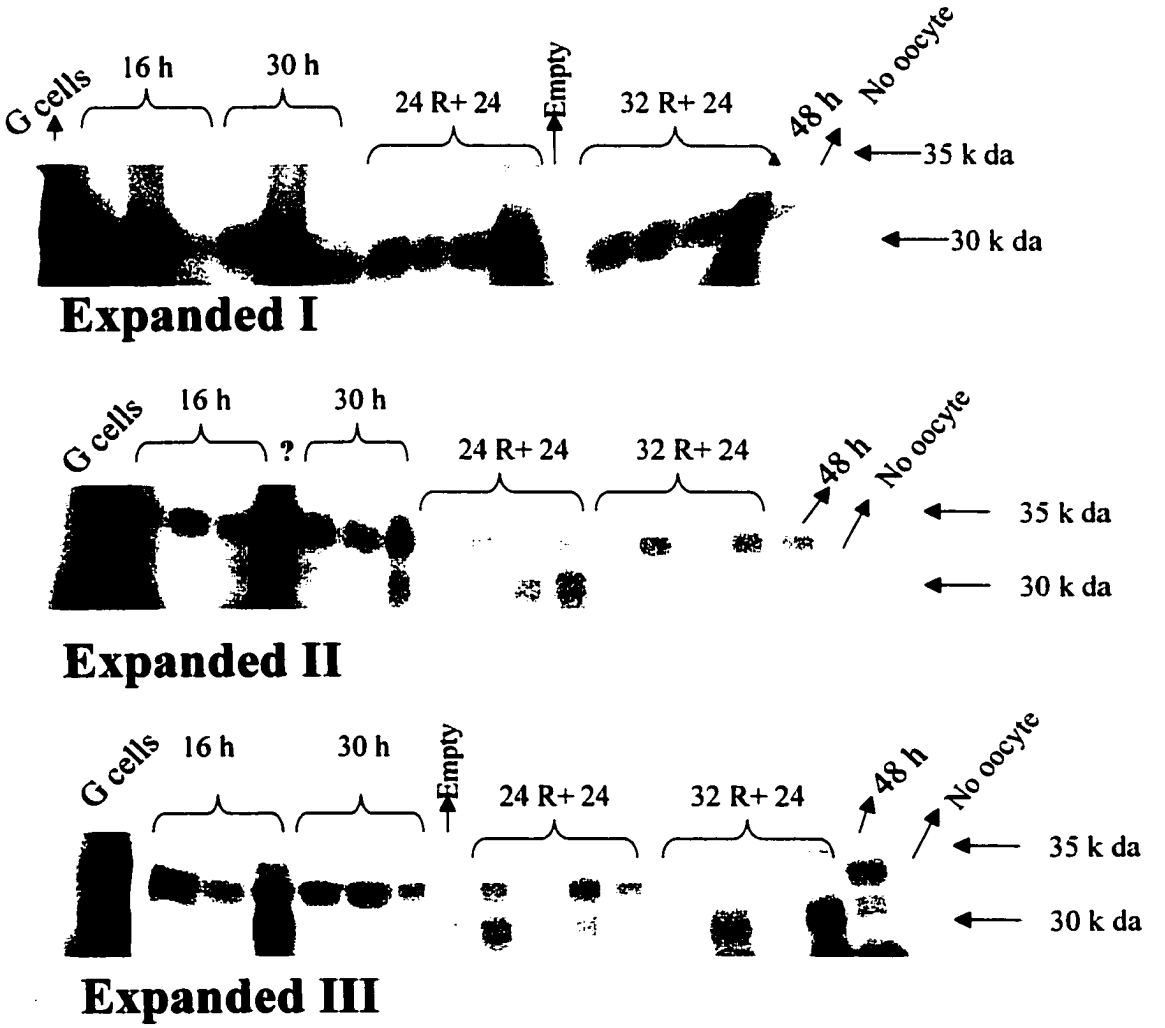



Figure 8 and 9- Histone kinase activity of equine oocytes with initial compact or expanded cumulus cells at different times in maturation (0, 16, 30, 48 h) and after being incubated with 66 μM roscovitine for 24 h and in maturation medium for additional 24 h (24 R+ 24) or incubated with 66 μM roscovitine for 32 h and in maturation medium for additional 24h (32 R+24). Individual oocytes were resolved in a SDS-PAGE gel and visualized by autoradiography. The intensity of each band was analyzed by the number of pixels with Image Quant® .The first lane of all gels corresponds to a positive control of granulosa cells. The last lane of each gel corresponds to a negative control where no oocyte was added.

**Figure 9-**



## CHAPTER IV

# Use of roscovitine for maintenance of the germinal vesicle stage in equine oocytes, and subsequent maturation and cleavage rates after intracytoplasmic sperm injection.

## INTRODUCTION

The effectiveness of roscovitine in preventing horse oocytes to spontaneously resume meiosis after removal of the follicle was demonstrated in chapter V. In this chapter, the potential of equine oocytes to develop into normal embryos after inhibition with roscovitine and subsequent incubation in permissive medium and intracytoplasmic sperm injection was evaluated.

## MATERIALS AND METHODS

### Collection of oocytes

Equine ovaries were collected at a local abattoir in Texas and rinsed in warm saline (35 to 37°C) upon collection. Five to 8 follicles ranging from 5 to 22 mm in diameter were randomly selected from each ovary. The follicular contents were scraped with a bone curette and washed into individual Petri dishes with 20 mM MOPS-buffered TCM199 (Sigma) at 35 to 37°C. Dishes were searched with the aid of a dissection microscope at 10 to 40 X, and oocytes were classified into expanded (Exp) and compact (Com) groups based on the appearance of the surrounding cumulus cells. Oocytes were

considered to be compact when most of the cumulus cells layers were tightly attached to the oocyte, and expanded when the cumulus cells surrounding the oocytes were loose, in a homogenous mass. Oocytes were placed in TCM199 with MOPS at 37°C and held for up to 60 min. Every 60 min, oocytes collected during that period were transferred to 5 ml Falcon tubes (Catalog # 352058, polystyrene) containing 1 ml of either maturation medium (Mat groups) or roscovitine-containing medium (roscovitine control and Ros+M groups; media are described below) covered with 2 ml of light paraffin oil, both previously equilibrated in 5% $CO_2$ in air. The Falcon tubes were transported to the laboratory in a Minitub portable incubator (Minitube of America, Verona, WI) at 38.2 °C. The total time from slaughter to incubation of oocytes in culture medium was 30 to 90 min. Upon arrival at the laboratory, oocytes were removed from the transport tubes and incubated in 500 µl of the same medium, in four-well multi-dishes (Nunc, Denmark) at 38.2 °C in 5% $CO_2$ in air. All components were purchased from Sigma unless stated otherwise. Base medium was a modified SOF (Maclellan et al, 2001 containing 0.8% BSA, 11 mM myoinositol, 10 µg/ml streptomycin sulfate and 1000 IU/ml penicillin G. Maturation medium was EMMI as described by Maclellan et al., 2001 consisting of the base medium with 15 ng/ml ovine FSH (NIDDK-oFSH-20), 1 µg/ml bovine LH (AFP-11743B) 1 µg/ml $E_2$, 500 ng/ml progesterone, 100 ng/ml EGF and 10 ng/ml IGF-1, with the addition of 10% fetal bovine serum (Gibco). Roscovitine-containing medium consisted of the base medium with 66 µM of roscovitine. Oocytes placed in roscovitine treatment were incubated for 24 h, and then a third of the total number were evaluated to determine the proportion of oocytes maintained in the germinal vesicle stage (roscovitine controls). These oocytes were denuded of cumulus by pipetting with a fine bore glass

pipette in a solution of 0.05% hyaluronidase in Hepes-buffered TCM 199 (Gibco Life Technologies Inc., Grand Island, NY) with 5 % FBS, fixed in 3.8% formaldehyde in 0.15M NaCl and stained with 2.5 μg/ml of Hoechst 33258 in 9:1 (v/v) glycerol:PBS, and observed under a E 600 Nikon fluorescence microscope with a 365 nm excitation filter to evaluate their chromatin configuration. Germinal vesicle chromatin was classified as condensed, intermediate, fibrillar, or diffuse, as previously described (Dell'Aquila et al, 2001). Other classifications included MI, MII and degenerating (abnormal chromatin or no chromatin visible). The remainder of the oocytes that had been incubated in roscovitine-containing medium were washed and transferred to 500 μl of maturation medium for 30 h at 38.2 °C in 5% $CO_2$ in air (Ros+M treatment). Oocytes not exposed to roscovitine treatments were incubated for either 30 h (30 h Mat) or 54 h (54 h Mat) at 38.2 °C in 5% $CO_2$ in air.

### Oocyte preparation for ICSI

After in vitro maturation, oocytes were denuded of cumulus as described above. Oocytes with a polar body were selected for ICSI. Oocytes without a polar body were fixed and stained as described above.

### Sperm preparation for ICSI

Fresh semen was collected from one stallion using a Missouri model artificial vagina, and then diluted to 80 x $10^6$ sperm/ml with a commercial skim milk glucose extender (EZ-Mixin, Animal Reproduction Systems, Chino, CA) before further processing. Semen was then frozen using a skim milk-egg-yolk based extender

(Blanchand et al., 1998). Semen straws (0.25ml) were thawed in 37°C water-bath for 30 sec. Two hundred μL of frozen thawed semen was placed in the bottom of a 5 ml tube containing 1 ml of modified TALP (sp-TALP, Parrish et al., 1988) and incubated in an atmosphere of 5% $CO_2$ in air for swim- up. After 20 min, 0.6 ml of medium was collected from the top of the tube, and centrifuged at 327 g for 3 min in a 1.7 ml polypropylene tube. The sperm pellet was resuspended and washed once with the same medium. The supernatant was removed, and the pellet was used for ICSI.

ICSI

Intracytoplasmic sperm injection was performed as described by Choi et al. (2002). The outside diameter of the sperm injection pipette was 7 – 8 μm. A 120 to 140 μm (outside diameter) pipette was used to hold oocytes. Immediately before injection, 1 μl of sperm suspension was placed in 3 μl of sperm TALP containing 10 % PVP (MW 360,000) under light white mineral oil. Sperm injection was carried out in a separate drop of Hepes-buffered TCM 199 containing 10% FBS. Each spermatozoon was immobilized by applying a few pulses with a Piezo drill (Prime tech Ltd., Ibaraki, Japan) to the sperm tail immediately before injection (Kimura and Yanagimachi, 1995). All manipulations were performed at room temperature. Injected oocytes were held for 20 min at room temperature in the same medium to heal the broken membrane slowly, transferred into G1.2 medium (IVF Science, Denver, CO, USA) in microdroplets under mineral oil, at a ratio of 5 μl medium per oocyte, and incubated at 38.2 °C in an atmosphere of 5% $O_2$, 5% $CO_2$ and 90% $N_2$ for 96 h.

### Sham injection

For the last 4 replicates, 25% of the oocytes having a polar body in the Ros+M and 30 h Mat treatments were subjected to sham injection. Sham injections were performed as described above, except that no sperm were in the injection pipette.

### In vitro embryo culture

After 96 h of in vitro culture, sperm- or sham-injected oocytes were fixed and stained as described above to examine the number and status of nuclei. Only nuclei that appeared to be normal were included in the number of nuclei recorded; nuclei with signs of degeneration (vacuolization, condensation, or fragmentation) were disregarded. Embryos were classified based on number of normal nuclei, as having 2 to 7, 8 to 16, and >16 normal nuclei within the cell.

### Statistical Analysis

Results with binomial responses were evaluated with chi-square analysis. Comparisons with expected values of less than 5 were analyzed using Fisher's exact test. The average number of embryonic nuclei was analyzed by ANOVA.

# RESULTS

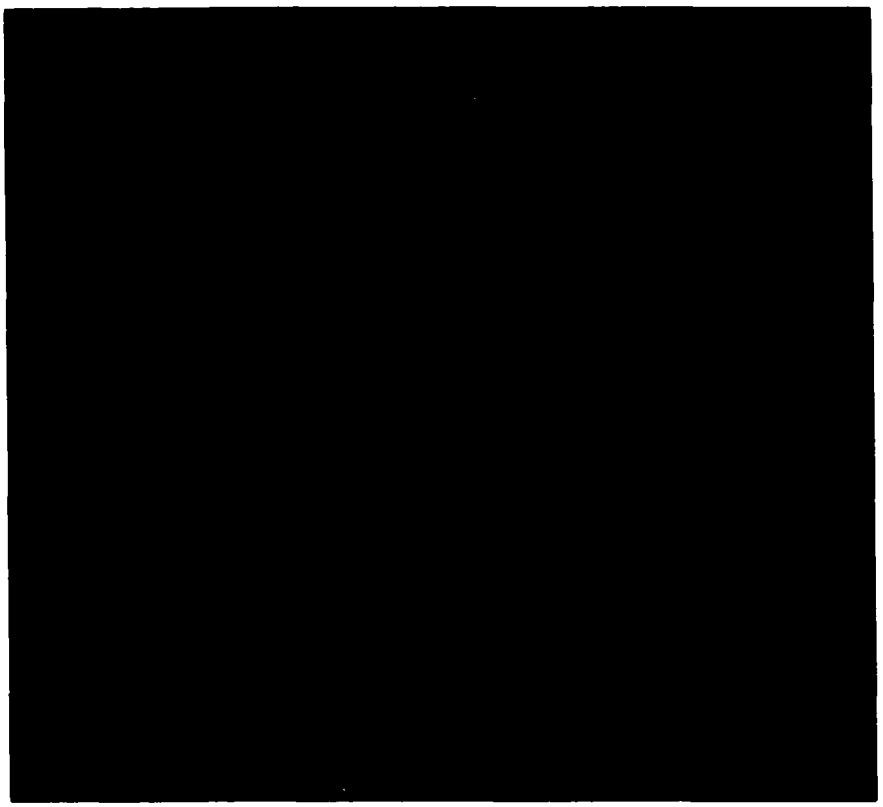
## Oocyte recovery and distribution

Out of a total of 211 ovaries processed, 1030 follicles were scraped (4.9 follicles per ovary), 611 oocytes were recovered (59% recovery rate) and 590 oocytes were used for this study. A total of 261 cumulus cell complexes (COC) were classified as Exp (44.2%), and 329 COCs were classified as Com (55.7%).

## Effect of roscovitine in meiotic suppression

Thirty-three Com and 28 Exp oocytes (5 replicates) were fixed and evaluated after 24 h of incubation with roscovitine (Ros-controls). A sixth replicate was disregarded due to a procedural error. Two Com oocytes fixed for evaluation were not analyzable due to presence of cumulus cells. The chromatin configurations seen in the analyzable Ros-control oocytes are presented in Table 1. Significantly more Com than Exp oocytes were in the GV stage, 26/31 (84%) vs. 16/28 (57%), respectively ($P < 0.05$). The 5 Com oocytes not in GV were degenerating. Of the 12 Exp oocytes not arrested in GV, 8 were in MI, 3 were in MII and 1 was degenerating. More Exp than Com oocytes were in MI ($P<0.05$). Figure 7 shows an oocyte in the germinal vesicle stage.

Figure 10 - Equine oocyte in germinal vesicle stage (arrow) after incubation with 66 μM or roscovitine for 24 h.



Stained with Hoechst
Magnification: 200X

In vitro maturation of oocytes

The proportions of oocytes in metaphase II (oocytes with a visible polar body plus those found to be in MII on fixation and staining) in the 30 h Mat, 54 h Mat and Ros+M groups are given in Table 2. There was no difference in maturation rates to MII among treatment groups. Over all treatments, a higher proportion of Exp than Com oocytes were in MII after maturation culture (167/203, 82% vs. 190/264, 72%, respectively; $P < 0.05$).

Of oocytes not in MII, 1 Com and 2 Exp oocytes were lost during fixation and staining. Of evaluated oocytes, a significantly higher proportion of Com than Exp oocytes were still in the GV stage after maturation culture (23/73, 32% vs. 3/34, 9%; $P < 0.01$). The percentage of oocytes in MI ranged from 0 to 4%. In the 30 h Exp group only 3 were degenerated and 3 were still in GV stages. Similarly, in the R-treated groups, a higher rate of degenerated oocytes was found in the Com than in Exp, 18/28 and 4/12, respectively. Also, 5/28 Com whereas no Exp, were still in the GV stage. Eight oocytes in the 30 h mat group (5 Com and 3 Exp) and 6 oocytes in the R treated (3 Com and 3 Exp) were in MI. Only 5 oocytes in the 54 h compact group were in MI.

Table 6- Chromatin configuration of equine oocytes evaluated after 24 h culture with roscovitine

| Oocyte Type | Number of oocytes evaluated | GV Stage (%) | Condensed chromatin (%) | Intermediate (%) | Fibrillar (%) | FN* (%) | MI (%) | MII (%) | Deg (%) |
|---|---|---|---|---|---|---|---|---|---|
| Compact | 31 | 26 (84)[a] | 20 (65) | 5 (16) | 0 | 1 (3) | 0[a] | 0 | 5 (16) |
| Expanded | 28 | 16 (57)[b] | 11 (39) | 0 | 4 (14) | 1 (4) | 8 (29)[b] | 3 (11) | 1 (4) |

*Fluorescent nucleus, homogenous fluorescence throughout nucleus
[a,b] Values within columns differ ($P<0.05$).

Table 7. Chromatin configurations of horse oocytes having compact (Com) or expanded (Exp) cumuli in three in vitro maturation treatments

| Maturation treatment | Oocyte type | n | GV (%) | MI (%) | MII (%) |
|---|---|---|---|---|---|
| 30 h Mat | Com | 117 | 15 (13) | 5 (4) | 78 (67) |
| 54 h Mat | Com | 46 | 3 (6) | 0 (0) | 37 (80) |
| Ros+M | Com | 101 | 5 (5) | 3 (3) | 75 (74) |
| Total | Com | 264 | 23 (9) | 8 (3) | 191 (72) [a] |
| 30 h Mat | Exp | 89 | 3 (3) | 3 (3) | 74 (83) |
| 54 h Mat | Exp | 40 | 0 (0) | 0 (0) | 30 (75) |
| Ros+M | Exp | 74 | 2 (2) | 1 (1) | 63 (85) |
| Total | Exp | 203 | 5 (2) | 5 (2) | 167 (82) [b] |

Ros+M; Oocytes were cultured in base medium with 66 μM roscovitine for 24 h and then cultured in maturation medium without roscovitine for additional 30 h.
[ab] $P<0.05$.

## Sham injection

Of 66 sham-injected oocytes, 65 (99%) survived injection. After 96 h culture, no cleavage was observed in sham-injected oocytes in the Ros+M treatment. In the 30h Mat treatment, 2/17 (12%) Exp oocytes and 3/18 (17%) Com oocytes cleaved after sham injection. Upon evaluation after staining with Hoechst, one embryo in the Ros+M Com group, which had been classified as uncleaved, was seen to have 2 normal nuclei. Of the

remaining 64 sham-injected oocytes, 45 contained one to multiple pronuclear-like structures, 1 was in MII, and the remainder were degenerating.

### Cleavage rates after ICSI

ICSI was conducted on 285 oocytes. After injection, 279 oocytes (98%) survived. The in vitro development of zygotes cultured for 96 h after ICSI is shown in Table 3. There were no differences ($P>0.05$) in morphological cleavage rates among treatments. The proportion of Com oocytes cleaving with normal nuclei was significantly higher in the Ros+M than in the 54 h Mat treatment ($P < 0.05$). The proportion of Exp oocytes cleaving with normal nuclei was higher in the 30h Mat than in the Ros+M treatment ($P<0.05$). Significantly more embryos developed to 8 or more cells in the 30 h Mat and Ros+M treatments than in the 54 h Mat treatment for Com oocytes, and in the 30 h Mat treatment than in the 54 h Mat treatment for Exp oocytes ($P < 0.05$). The average number of nuclei was higher ($P<0.05$) in Ros+M Com oocytes (13.5) than in any other group (3.6 to 9.3).

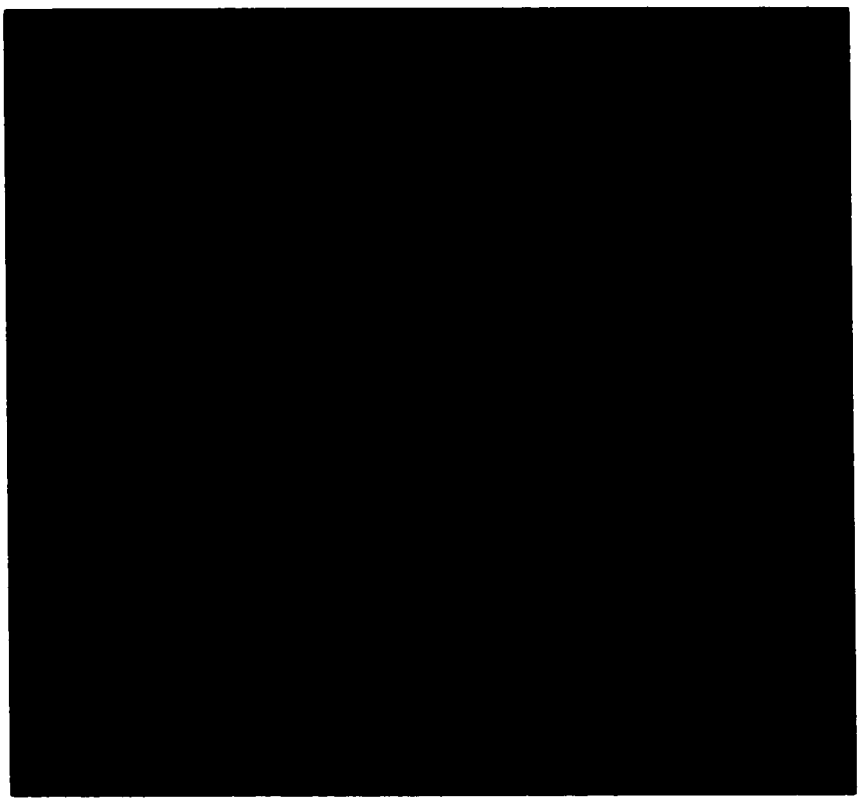
Table 8. Subsequent in vitro development of horse oocytes treated with roscovitine for meiotic suppression and fertilized by intracytoplasmic sperm injection

| Maturation treatment/ oocyte type | n | Oocytes cleaved (%) | Embryos with normal nuclei (% of n) | Nucleus number (% of embryos with normal nuclei ) | | | Average number of nuclei (mean ± SEM) |
|---|---|---|---|---|---|---|---|
| | | | | 2-7 n | 8-16 n | >16 n | |
| 30 h Com | 55 | 45 (82) | 30 (55) [a,b] | 15 (50) [a] | 10 (33) | 5 (17) | 9.3 ± 1.4 [a] |
| 54 h Com | 36 | 30 (83) | 13 (36) [a] | 12 (92) [b] | 1 (8) | 0 (0) | 4.8 ± 0.7 [a, b] |
| Ros+M Com* | 56 | 45 (80) | 35 (63) [b] | 13 (37) [a] | 13 (37) | 9 (26) | 13.5 ± 2.1 [c] |
| 30 h Exp | 54 | 41 (76) | 34 (63) [b] | 19 (56) [a] | 12 (35) | 3 (9) | 8.7 ± 0.9 [a] |
| 54 h Exp | 29 | 18 (62) | 13 (45) [a,b] | 12 (92) [b] | 1 (8) | 0 (0) | 3.6 ± 0.9 [b] |
| Ros+M Exp | 48 | 34 (71) | 20 (42) [a] | 13 (65) [a,b] | 6 (30) | 1 (5) | 7.2 ± 1.2 [a, b] |

*Ros+M; Oocytes were cultured in base medium with 66 μM roscovitine for 24 h and then cultured in maturation medium without roscovitine for additional 30 h.

[a,b,c] Values in the same column within cumulus types without common superscripts differ significantly ($P<0.05$)

Figure 11- Equine embryo with 16 normal nuclei (and 3 mitotic figures) produced from a oocyte in the compact group, 96 h after ICSI.



Stained with Hoechst
Magnification: 400X

## DISCUSSION

Results of this study show that roscovitine was effective in maintaining equine oocytes in the germinal vesicle stage, without a decrease in meiotic or developmental competence, but that there was a differential effect depending on oocyte type. The method used in this study to classify cumulus cell complexes (COC) was based on the expansion of the cells directly surrounding the oocytes, without regard to granulosa cell

morphology. Other studies have used both mural and cumulus granulosa for oocyte classification (Hinrichs et al., 2002, Choi et al., 2002c). The proportion of compact COC has been around 50% (Hinrichs et al, 2000) and 45% (Hinrichs et al, 1993b; Alm et al, 1996) when based on cumulus morphology alone, but 33 to 36% and 37% (Hinrichs et al., 2002, Choi et al., 2002c) when based on both mural and cumulus granulosa. In this study, 56% of oocytes were classified as Com.

Roscovitine inhibits ATP binding to the p34 cdc2 subunit of MPF. It is effective at 25 μM to 100 μM in maintaining around 80% of bovine oocytes in GV for 24 h without affecting the developmental potential, while the lower dose of 12.5 μM only maintained 60% of oocytes in the GV stage (Mermillod et al, 2000). In our preliminary studies with equine oocytes, roscovitine was effective in maintaining the GV for 24 h in 73% of Com and 38% of Exp at 66 μM; and 65% Com and 55% Exp at 200μM, in media without hormones (Franz et al, 2002). In the present study, the 66 μM dose was chosen to ensure that the treatment was reversible, and to reduce the chance of affecting subsequent developmental competence. Our results show that this dosage maintained 84% of Com and 57% of Exp oocytes in the GV stage after 24 h of incubation. We did not determine the proportions of oocytes in the GV stage at the time of recovery; however, previous studies have reported that this proportion is similar for oocytes with compact and expanded cumuli (Zhang et al, 1989, reported 28 and 23%, respectively; Hinrichs et al, 1993 a, 78 vs. 73%, respectively; Hinrichs et al, 1995; 68 and 69%). Therefore, we assume that the majority of the oocytes were in GV stage right after collection. The results for meiotic suppression of Com oocytes were similar to those described for bovine oocytes (88% at 50 μM-Mermillod et al, 2000) and porcine oocytes (70-74%, 80 μM and

120 μM; Ju et al, 2001). Interestingly, 20/23 (86%) Com and 11/26 (68.5%) Exp of the GV oocytes had condensed chromatin configuration, which has been attributed to the configuration with highest developmental potential in vitro (Hinrichs et al, 1997). In most species, oocytes surrounded by expanded cumulus cells at the time of recovery from abattoir ovaries are discarded; therefore, equine expanded oocytes cannot be easily compared to those of any other species. In the horse, expanded oocytes have been reported to have higher rates of in vitro maturation (Hinrichs, 1995 and 1997) and to mature more rapidly in vitro than do oocytes derived from compact oocytes (Hinrichs et al, 1993; Zhang et al, 1989). Expanded oocytes also require a shorter period for protein synthesis (Alm and Hinrichs, 1996) before commitment to meiosis. A small proportion of expanded oocytes, those from the most atretic follicles, are already in MI or MII at the time they are recovered from the follicle (Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000). Taken together, these results suggest that a proportion of expanded oocytes are already maturing, or committed to mature if removed from the follicle; thus roscovitine is less effective in suppressing meiosis in this population than in compact oocytes.

The IC50 value for roscovitine to inhibit in vitro kinase activity is 0.45μM (Meijer et al, 1997). Some authors believe that higher doses actually are more effective because they also interfere with erk 1 and erk 2, consequently affecting the MAPK cascade (Ponderato et al, 2001). In the horse, the only study that evaluated MAPK activity concluded that MAPK is present in immature and mature oocytes in the form of erk 2 (Goudet et al., 1998). It is not clear if erk 1 is not present or if it is present at undetectable levels. It has been hypothesized that in immature (incompetent) equine

oocytes, MAPK remains un-phosphorylated, whereas in competent, partially competent and preovulatory oocytes it becomes phosphorylated with a high kinase activity (Goudet et al, 1998). Therefore, perhaps higher doses of roscovitine would have better blocked the action of MAPK and been more effective in maintaining meiotic arrest in the Exp group.

The suppressive action of roscovitine in Com oocytes in the current study is in contrast to the report of Hinrichs et al. (2002), in which 24 h culture with roscovitine maintained only 16 to 34% of compact oocytes in the GV stage. In that study, suppression was related to the chromatin configuration within the GV. Oocytes having condensed chromatin were capable of being suppressed, whereas oocytes having diffuse chromatin degenerated during culture. A major difference between these two studies is the delay from slaughter to oocyte recovery. In the present study, oocytes were placed into culture between 30 and 90 min after slaughter, whereas in the previous report, including time for ovary collection and transport, this period was 3 to 8 h. Oocytes with diffuse chromatin appear to undergo degeneration during ovary storage (K. Hinrichs and Y.H. Choi, unpublished data); thus, the oocytes that failed to respond to roscovitine treatment in the previous study may have begun to degenerate before culture was initiated.

The chromatin configuration within the GV may have changed during roscovitine treatment in the present study. While the initial chromatin configuration of the oocytes was not determined, previous studies at the same abattoir have shown that in oocytes fixed within 45 min of recovery from the follicle, diffuse chromatin was found in 62% of oocytes with compact cumuli, and only 4 % of oocytes with expanded cumuli; the proportions of condensed chromatin in these two oocyte types were 36% and 62%,

respectively (K. Hinrichs, unpublished data). In the present study, after roscovitine treatment 20/23 (86%) Com and 11/26 (69%) Exp oocytes had condensed chromatin within the GV. These findings suggest that during the time that roscovitine maintained meiotic suppression, chromatin condensation occurred in Com oocytes. Further studies examining chromatin configurations of horse oocytes before and after roscovitine treatment, would be needed to confirm this hypothesis. Roscovitine treatment did not affect the meiotic competence of the suppressed oocytes, as there was no difference in maturation rates between the Ros+M treatment and either 30 h or 54 h maturation controls.

The overall maturation rates to MII in our study were higher than typically reported for equine oocytes, which could possibly be related to their rapid placement into culture (Dell'Aquila et al, 1997b, 2001), but were lower than those reported by Dell' Aquila et al, 1997b (83% for Com and 89% for Exp) when a comparable time from slaughter to culture and incubation time was used. Considering that the time of the year was similar in both studies, this could be attributed to the different collection systems used: in our study follicles were scraped with a bone curette while the reference cited used aspiration. In contrast, in a study comparing scraping vs. aspiration (Dell'Aquila et al, 2001) no differences were found in MII rates, but rates were lower than the ones reported previously by the same author (55.4% for scraped and 61.4% for aspirated). Scraping the granulosa layer in the follicle results in a recovery of more cumulus-intact oocytes (Dell' Aquila, 2001). The population of oocytes recovered by aspiration results in more oocytes at advanced stages of atresia that are often free within the follicle antrum, while in scraping those oocytes are lost (Mlodavska et al, 1997).

Results in nuclear maturation of equine oocytes are difficult to compare among laboratories due to the great variability of procedures used. Since 1981, most workers in this field agree that equine oocytes require more than 24 h and less than 40 h of incubation in vitro to reach MII (Fulka et al, 1981). Maturation times of 24 h for expanded COC and 30 to 36 h for compact COC were reported (Zhang et al., 1989; Hinrichs et al, 1993) and have been generally accepted. To facilitate comparison and statistical analysis, we used 30h for both groups of COC. However, now that ICSI is available as a method for fertilization of horse oocytes, more work is needed to define the effect of duration of in vitro maturation on developmental competence of both types of oocytes.

The 54 h Mat treatment in this study served as a control for the Ros+M group since it would be incubated for the same total period as the combined roscovitine culture and subsequent maturation. Results of in vitro maturation for times longer than 36 h have been reported by Fulka et al, 1981 (68.2% MII after 40 h); Choi et al, 1993 (67% MII after 40 h); Shabpareh et al, 1993 (84.2% MII after 48 h); Sonowski et al, 1997 (74.4% after 42h) and Del Campo et al, 1995 (47% MII after 48 h). Nuclear maturation in vitro for equine oocytes plateaus after 32 h (Del Campo 1995; Choi et al, 1993) with no further increase in MII rates. In this study, there was no difference in maturation rates among groups examined.

Most of the remainder of oocytes that were not in MII at the end of the culture time from the Com groups, were in GV (36% – 30Mat, 33%- Ros). Surprisingly, the great majority of oocytes in the 30 h Com group, had a CC configuration (10/41), 3 had Fibrinous and 2 had Tangled configurations. No fluorescent chromatin (FN) was

detected. In the 54 Mat Com and Ros Com 1/3 and 3/5 oocytes were classified as condensed chromatin CC. It has been suggested that the condensed chromatin configuration represents the meiotically competent oocyte in the equine species, and that oocytes with FN nuclei do not appear to contribute to the population of oocytes that mature in culture (Hinrichs et al, 1997). FN chromatin was associated with 50% of oocytes derived from follicles <20mm in diameter (Hinrichs et al, 1997b and Hinrichs et al, 2000). Perhaps, the control medium used for this study was supportive enough of maturation and was able to rescue the population of oocytes (to CC) that would normally be characterized as FN, but was not able to take them any further in the meiotic process.

We performed sham injection to evaluate the extent of parthenogenetic activation induced by the ICSI procedure. While some treatments showed morphological cleavage in a small proportion of oocytes (12 to 17%), the only apparently normal nuclei were visualized in one oocyte, which had not cleaved. Data on sham injection are limited in the horse. Grondahl et al, 1997; included a sham injection treatment in their study on ICSI, finding no sign of activation (pronucleus formation, $2^{nd}$ polar body extrusion, fragmentation or cleavage) after 20 h of incubation. The most common chromatin configuration in sham-injected oocytes in the present study was one to multiple pronucleus-like structures. We cultured the oocytes for 96 h after injection; this long culture period could have predisposed the oocytes to spontaneous "activation", i.e., formation of pronuclei from multiple or individual chromosomes.

Cleavage rates after ICSI in the horse are difficult to compare since most reports only discuss visual (morphological) cleavage, without assessing nuclear status. Based on morphological cleavage alone, cleavage rates in this study varied between 62 and 83%,

which is comparable to other recent reports in the horse (Li et al, 2001; Choi et al, 2002a). Studies on in vivo embryonic development in the horse demonstrated that at 48 h post-ovulation, embryos had 4 to 6 cells; at 72 h, 7 to 11 cells were detected, and at 96 h embryos had from 8 to 16 cells (Bezard et al., 1989; Betteridge et al., 2000). We considered cleavage with fewer than 8 normal nuclei after 96 h of culture to indicate retarded development. The treatment with the largest proportion of embryos in that range (2 to 7 nuclei) was 54 h Mat. This group had nuclear maturation and morphological cleavage rates similar to the other treatments; however, the rate of cleavage with normal nuclei was lower than for the 30 h Mat for Exp or Ros+M for Com, and the proportion of embryos arrested before 8 cells was significantly higher. Thus, the developmental potential of both Com and Exp oocytes appeared to be compromised by the extended maturation time. Interestingly, Exp oocytes in the Ros+M group appeared to perform intermediately between the 30 h Mat and 54h Mat groups, suggesting that for Exp oocytes, the increased cytoplasmic maturation time allowed during roscovitine treatment was not helpful, and may in fact have resulted in over-maturity and loss of developmental potential.

The highest average number of nuclei per embryo was achieved in the Ros+M Com group; this was higher ($P<0.05$) than for any other treatment. This indicated that culture in the presence of roscovitine probably allowed an increase in developmental competence (cytoplasmic maturation) within Com oocytes, while maintaining meiotic suppression.

The extent of embryo development after ICSI has been variable among laboratories: Grondahl et al. (1997) reported 8% of embryos had cleaved to >8 cell after 72 h of culture and Maclellan et al. (2000) reported 5% cleavage to 9 to 32 cells range

and 5 % >32 cells after 144 h (6 day) of culture. Guignot et al. (1998) reported 46% cleavage after 2 days of culture; after 5 days of culture, 12% of embryos had 9 to 16 cells and two embryos cleaved to 50 to 80 cells. Dell'Aquila et al. (1997b) reported that 39% of embryos with 6 to 8 cells after 6 days of culture. Results are difficult to compare since the starting points (oocytes injected vs. cleaved embryos) endpoints, methods of culture and methods of assessment vary among studies. In the present study, the average number of 14 nuclei in the Ros+M Com group compares favorably with embryo development in vivo as described above, and to development of embryos recovered from the oviduct 96 h post-ICSI with frozen-thawed sperm (14 nuclei, Choi et al., 2002a).

In conclusion, culture with roscovitine maintained equine oocytes in the GV stage efficiently for 24 h, and was more effective in oocytes originally having a compact cumulus than in those having an expanded cumulus. Suppression with roscovitine was reversible; it did not affect meiotic competence after subsequent maturation in vitro for either cumulus type. In oocytes originally having a compact cumulus, 24 h culture with roscovitine before maturation significantly increased oocyte developmental competence after fertilization. However, culture with roscovitine before maturation significantly decreased rates of normal cleavage after fertilization in oocytes having an expanded cumulus. In future studies with roscovitine with equine oocytes, it would be highly desirable to determine pregnancy rates and normality of offspring after transferring resulting embryos to the reproductive tract.

# CHAPTER V

## CONCLUSIONS

The experiments described in this dissertation resulted in the following conclusions:

1) Roscovitine was effective in maintaining equine oocytes in the germinal vesicle stage for up to 32 h at concentrations of 66 and 200 μM, but not at 22 μM.
2) Roscovitine was more effective in maintaining the GV stage in oocytes that had compact surrounding cumulus cells at the beginning of incubation than those with expanded cumulus cells.
3) The chromatin configuration of germinal vesicle stage observed in oocytes blocked with roscovitine (compact chromatin) has been described as the most competent morphology for equine oocytes in vitro.
4) The meiotic block induced by roscovitine in equine oocytes is reversible and maturation can be completed in vitro after release of suppression and incubation in permissive medium
5) The levels of MPF activity based on the histone kinase activity assay for oocytes inhibited with roscovitine and allowed to complete maturation afterwards were similar to oocytes in control groups.

6) Oocytes pre-incubated with 66 μM of roscovitine and allowed to complete maturation after inhibition were capable of normal fertilization and cleavage after ICSI.

7) Use of roscovitine did not improve or decrease maturation rates or cleavage rates in equine oocytes

8) Embryos resulting from ICSI of oocytes with compact cumulus cells, pre-treated with 66 μM of roscovitine, had more nuclei than any other group.

9) Further studies on equine oocyte maturation should investigate the extent of the benefit of adding roscovitine in pre maturation systems, by determining in vivo viability after transfer of resulting embryos to the reproductive tract of mares.

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# APPENDIX

## EFFECT OF TIME AND TEMPERATURE DURING OVARY TRANSPORT ON NUCLEAR AND CYTOPLAMIC MATURATION OF EQUINE OOCYTES

## INTRODUCTION

Time of ovary transport after slaughter affects maturation rates and competence of the oocyte in vitro. According to Hochi et al (1993), storage of equine ovaries for 3, 6 and 9 h at 30°C resulted in 62.5%, 45 % and 30 % oocytes in MII. In contrast, Shabpareh et al (1993), evaluated cumulus expansion and maturation of equine oocytes kept at 25°C for 5-6, 6-7 and 7-8 h after slaughter and found no difference between groups. Similarly, Del Campo et al (1995), compared the times of 3-9 and 10-15 h at 30° C to 35°C, and no effect on maturation (49% MII) was observed after 24 h in culture.

Short term storage of ovaries (1.5 to 4 h) and long term storage before oocyte collection (6 to 8.5 h) at 37°C was described by Guignot et al (1999). After 30 h of maturation, there was no difference on MII rates, but a higher percentage of oocytes had membrane damage in long term storage when oocytes initially had a compact cumulus morphology. It was also observed that the recovery rates were higher after longer storage.

Recently, Pedersen at al, (2000), demonstrated that temperature had no influence on chromatin configuration, but influenced cumulus morphology, with more expanded cumulus cells at lower temperatures (20°C). These results seem to be in conflict with our observations, since cumulus morphology is highly related to nuclear oocyte competence

(Hinrichs et al, 1997; 2000). A change in chromatin configuration was observed from 4 to 6 h of storage, but the results did not show an effect of temperature.

In bovine oocytes, the rate of maturation increases under post-mortem conditions, but storage of ovaries for prolonged times may affect blastocyst formation. Yang et al (1990), demonstrated that storage of ovaries at 37°C is detrimental for cleavage rates and blastocyst formation at 8, 16 and 24 h. At 25°C there was no difference between times examined (0, 4, 8, 16, 24 h), and at 4°C there was a lower percentage of cleavage and much lower (0 to 1.6%) blastocyst formation at times 4 to 24 h. Controversially, Solano et al (1994), demonstrated that storage of bovine oocytes for 12 to 24 h at 4°C did not affect the maturation or the cleavage rates.

Cortical granules are secretory granules processed in the Golgi (Hyttel et al, 1986). They are composed of enzymes and glycoproteins that cause changes to the zona pellucida that cause for the block to polyspermy upon fertilization (Cran et al, 1989). Changes in numbers and distribution of cortical granules have been described in mouse (Ducibella et al, 1988), pig (Wang et al, 1997), bovine (Izadyar et al, 1998) and horse (Carneiro et al, 2002) oocytes, and have been associated with maturity of the cytoplasm. The potential of an oocyte includes meiotic competence (ability to resume meiosis and complete maturation to MII) and developmental competence (ability of a fertilized oocytes to produce a viable embryo).

The effect of time and temperature has not been correlated with cortical granule migration that is associated with nuclear maturation in the equine. Under our laboratory conditions, we see a decrease in oocyte quality with increasing time between slaughter and retrieval of oocytes.

The objective of this study was to evaluate the effects of time and temperature during transport of equine ovaries after slaughter on nuclear maturation and cortical granule migration to optimize success rates.

## MATERIAL AND METHODS

Ovaries collected at the slaughterhouse were rinsed several times with saline at 29°C, and grossly trimmed. These ovaries were divided into three groups: GI was processed within 1 h after slaughter, and GII and GIII were placed in zip lock bags identified with time of collection, and randomly divided into two transport temperatures: 16 to 18°C or 27 to 29°C. Follicles 8 to 25 mm in diameter were cut individually with a scalpel blade into a 35 mm petri dish and the internal follicular walls were scraped with a bone curette and rinsed with TCM 199 buffered with 20 mM MOPS. Cumulus-oocyte complexes (COC) were classified based on expansion of the follicular cells into expanded and compact (Hinrichs and Williams, 1997). After this classification, oocytes were further classified into expanded and compact based on the COC appearance. Therefore the classifications were EE (expanded expanded) EC (expanded compact) and CC (compact compact).

Ovaries in GII and GIII were processed upon arrival at the laboratory, and oocytes were placed in culture medium (5% $CO_2$ at 38.5°C), 6 to 9 h after slaughter. Oocytes recovered in GI were placed in 5 ml tubes containing pre-equilibrated maturation medium covered with mineral oil; maturation was started in a portable incubator regulated at 39°C. Upon arrival, oocytes were removed from the tubes, washed, and placed in 500 µl

of EMM1 (a modified SOF) with 15 ng/ml FSH, 1 μg/ml bovine LH, 1 μg/ml $E_2$, 500 ng/ml progesterone, 100 ng/ml EGF, and 10 ng/ml IGF-1, and 10% BSA and incubated for 22 to 26 h in 5% $CO_2$ at 38.5°C. Oocytes from the transported ovaries were incubated in similar conditions.

After maturation, oocytes were stripped of their surrounding cumulus cells with 300 IU of hyaluronidase, washed, fixed in 4% paraformaldehyde for 20 minutes, permeabilized in 0.1% Triton X for 6 –10 minutes, washed, and stained in the dark with Hoechst 33342 (25μg/ml) for 6 -10 min to evaluate nuclear maturation; they were also stained with FITC – PNA (10μg/ml) to evaluate cortical granule migration. Cortical granule migration was graded from I to V. In oocytes with grade I, there was halo of cortical granules in the periphery and no granules in the central region of the oocytes and no retraction of the periviteline space; II - there was a halo of cortical granules in the periphery and few granules in the central region of the oocytes with little retraction of the periviteline space; III - granules were evenly distributed in the oocyte cytoplasm with no evidence of a halo and no retraction of the periviteline space; IV- granules were mostly in a halo but severe retraction of the periviteline space was noted; V- granules were distributed in the oocytes in clusters, and the periviteline space was severely retracted. Oocytes were mounted on slides and evaluated with laser scanning confocal microscopy. Cortical granule migration was considered positive in oocytes with grades I to II. The experiment was repeated three times, and data were analyzed by ANOVA with the arcsin transformation.

## RESULTS

A total of 90 EE, 76 EC and 38 CC oocytes were analysed. Results for nuclear maturation rates (MII), and cortical granule migration are presented in Appendix Table I. More EE oocytes matured to MII ($P<0.05$) than EC or CC oocytes. EE oocytes matured to MII similarly (63 to 76%) regardless of transport method, and all types of oocytes matured well (59 to 65% of MII) if placed in culture within 1 h of slaughter. Maturation rates were poor (20 to 45%) for EC and CC oocytes from ovaries transported 5 to 9 h at both temperatures studied.

The EE oocytes had the highest incidence of grades I and II of cortical granule migration whereas the CC had the highest incidence of grades IV and V. Cortical granule migration showed a similar pattern of maturation to meiosis II except that migration was poor for all CC oocytes. There was a positive relationship between nuclear maturation and cortical granule migration ($r^2 = 0.47$, $P < 0.05$).

Appendix Table 1- Effect of transport time and temperature on maturation rates of equine oocytes.

| Oocyte type | <1 h @ 20 to 26°C MII (%± SE) | 5 to 9 h @ 15 to 18°C MII (%± SE) | 5 to 9 h @ 27 to 29°C MII(%± SE) | Main effect mean MII(%± SE) |
|---|---|---|---|---|
| EE | 63 ± 12 | 76 ± 12[a] | 73 ± 12[a,c] | 71[a] |
| EC | 68 ± 12 A | 28 ± 12[b] B | 42 ± 12[d] AB | 46[b] |
| CC | 59 ± 12 A | 51 ± 15[a,b] AB | 20 ±12[b] B | 43[b] |

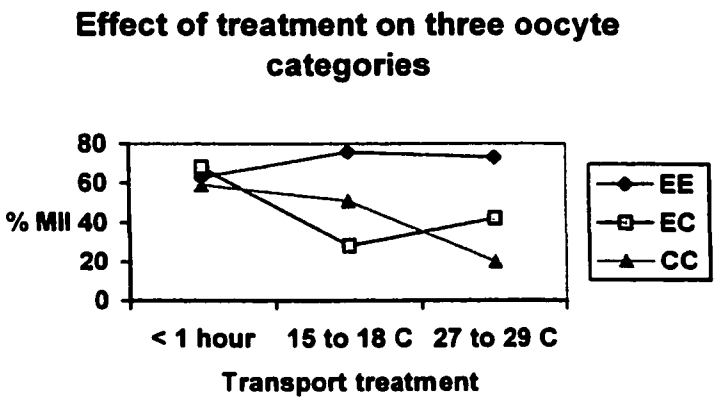
[a,b] Means within the same columns without common superscripts differ ($P<0.01$)
[c,d] Means within the same columns without common superscripts differ ($P<0.1$)
[A,B] Means within the same row without common superscripts differ ($P<0.05$)

Appendix Table 2- Effect of time of transport time and temperature in cortical granule migration of equine oocytes.

| Oocyte type | <1 h @ 20 to 26°C CGM (%± SE) | 5 to 9 h @ 15 to 18°C CGM (%± SE) | 5 to 9 h @ 27 to 29°C CGM (%± SE) |
|---|---|---|---|
| <1 h @ 20 to 26°C | 38 ±10 [a] | 41 ±10 [a] | 33 ± 10 [a] |
| <1 h @ 20 to 26°C | 43 ±10 [a] | 13 ±10 [a] | 27 ± 10 [a] |
| <1 h @ 20 to 26°C | 22 ±10 [a] | 20 ±12 [a] | 0 [a] |

No significant differences (P>0.05)

**Appendix Fig I –**




## DISCUSSION

Generally, it has been accepted that transport of equine ovaries up to 6 h before oocyte collection does not affect the viability of the oocytes for in vitro procedures (Hochi et al, 1993; Choi et al, 1993; Hinrichs et al, 2000). Although blastocysts have been produced with equine ovaries transported for 24 h (Galli et al, 2000), our results indicate that conditions of transport can affect oocyte maturation in vitro, but this is dependent on the type of oocyte. The morphology of the cumulus-oocyte complexes has been related to follicle size, atresia, developmental capacity and chromatin configuration.

been related to follicle size, atresia, developmental capacity and chromatin configuration. Expanded oocytes are known to be more competent for completing meiotic maturation that compact oocytes, and they also require less time in maturation (Zhang et al, 1989; Hinrichs et al, 1993, Alm and Hinrichs, 1996). The criteria used to define expansion and compaction of the cumulus differs between laboratories, and the methods used to recover the oocytes can affect the integrity of the cumulus (Alm et al, 1997). Equine oocytes are embedded in the follicular wall (Hawley et al, 1995), and therefore when aspiration is used as the collection method for slaughterhouse ovaries, a great proportion of the oocytes recovered are the ones floating the follicular fluid in advanced stages of atresia (Mlodavska et al, 1997). When scraping is used to collect oocytes less than 10% of the oocytes are denuded, and similar proportions of expanded and compact COCs are recovered (Hinrichs et al, 1993; Alm and Hinrichs, 1996; Hinrichs and Williams, 1997). The double method of classification for cumulus expansion used in this study was based first on the classification system proposed by Hinrichs and Williams, (1997), in which each individual follicle is scraped in a dish and expansion of the follicular cells in addition to expansion of the cumulus cells directly surrounding the oocytes are considered. The second evaluation used in this classification system was based on the expansion of the cumulus cells immediately surrounding the oocytes, a more traditional method. In this study, there was an even distribution of oocytes in the EE and EC classes for all collection times, and significantly fewer oocytes in the CC category. This indicates that in most studies, the oocytes in the EC category are distributed between the EE and the CC classes. In this study, EE oocytes had the highest MII and CG rates regardless of time of transport, which corresponds to the most competent oocyte type. The results also

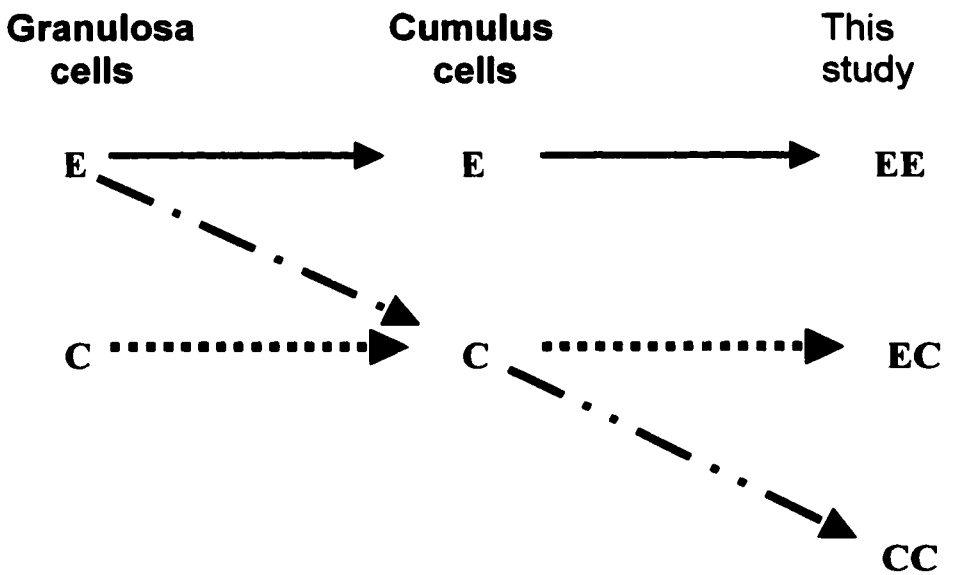
suggest that the EC class of oocytes resulted in better maturation rates when transported in 27 to 29°C, and that the CC class had a slight advantage in 15 to 18 °C. Oocytes with expanded cumulus cells at the time of collection require less time in maturation than those with the compact classification, so perhaps higher maturation rates of the EC could be achieved if they would be incubated for a slightly longer period of time. The CC oocytes in this study were correlated with the lowest MII rates and cortical granule migration grades. These oocytes are probably derived from smaller follicles, probably have less competent cell systems to mature, and therefore the conditions and time for in vitro maturation used in this experiment were not sufficient to stimulate them to resume meiosis.

Mlodavsaka et al (1997), demonstrated that most oocytes derived from follicles between 4-10 mm in diameter had started the atretic process. A certain level of atresia is beneficial for oocytes matured in vitro (Hinrichs and Williams, 1997), but oocytes with severe atretic characteristics usually degenerate if cultured. Results of this study suggest that the EE class is probably the most atretic and, therefore, are the easiest to mature in vitro. The EC class has probably started atresia but the inhibitory effects in the follicle were still present, therefore a longer time in culture would be beneficial to complete maturation to MII. This is supported by the study of Hinrichs and Williams (1997), in which less than 10% of the oocytes with no signs of expansion (in cumulus or granulosa cells) were in 2nd or 3rd degree of atresia.

The dual standard used in the classification method of this study can benefit the selection of oocytes with the least developmental competence since the ability to identify accurately degenerate changes in oocytes before culture is initiated is still limited.

In agreement with the recent study by Hinrichs et al, (2002), we concluded that the best maturation rates for equine oocytes collected from slaughterhouse ovaries are achieved when oocytes are placed in maturation medium within one hour of slaughter; but if ovaries need to be transported, temperatures of 16 to 18 °C are recommended.

Appendix Figure 2- Three ways of classifying equine oocytes by cumulus oophorus cell morphology




The most common approach used by researchers is to classify the cumulus cells surrounding the oocyte as compact or expanded (sometimes degree of expansion), middle column of Figure 2. Hinrichs et al, frequently used the scheme on the left. In this study, both systems were combined; note that the CE category does not exist and also that the E category for granulosa cells could be either C or E for cumulus cells.