RESEARCH ON FELINE IN VITRO MATURATION AND THE CELL CYCLE

A Senior Honors Thesis

By

ASHLEY MICHELLE COX

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

APRIL 2000

Group: Cell Biology I

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For the Designation of

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Approved as to style and content by:

Duane C. Kraemer
(Fellows Advisor)

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(Executive Director)

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ABSTRACT

Research on Feline In Vitro Maturation

and the Cell Cycle. (April 2000)

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The feline meiotic cycle is poorly understood. In order to elucidate the events occurring during meiosis in the cat oocyte, a study of the levels of Maturation Promoting Factor (MPF) and MAP Kinase (MAPK), enzymes thought to be necessary to induce oocyte maturation, is important. However, before MPF levels can be studied, a method for maturing feline oocytes *in vitro* efficiently must be perfected. Furthermore, the information gathered from this study will be beneficial for research on the conservation of endangered feline species and for other reproductive techniques.

This study focused on identifying an efficient method of maturing feline oocytes *in vitro* and observing MPF and MAPK levels at different stages of maturation and after activation in the feline oocyte.

A study of the comparative efficiencies of different maturation media was conducted using feline oocytes collected from spayed feline tracts. Three different previously published maturation medium protocols were chosen and compared to determine which

produced the highest rate of maturation. As a control, oocytes were put into the base maturation medium without the addition of hormones. The oocytes were incubated for 24 hours in humidified 5% CO_2 in air. The oocytes were scored to determine the stage of meiosis they had achieved--germinal vesicle stage, metaphase I, or metaphase II. Only oocytes reaching the metaphase II stage were considered matured. The results of this experiment showed that increasing hormone concentrations in the culture medium increases the rate of feline oocyte maturation.

To study the cell cycle and MPF and MAPK activity during meiosis, oocytes from the germinal vesicle, metaphase I, and metaphase II stages of meiosis were collected after maturation in the medium determined to be most efficient, as well as activated oocytes. Activated oocytes were obtained by activating mature oocytes with an ionomycin/cycloheximide treatment. Levels of MPF and MAPK activity at different stages in the feline cell cycle were determined by SDS-PAGE after MPF and MAPK reactions.

The results of this study show that MPF and MAPK activities are low at the germinal vesicle stage of meiosis and high at both metaphase I and metaphase II in the feline oocyte. Furthermore, the activity of MPF and MAPK decreases after activation until the cell starts mitotic divisions when the levels start to rise again.

ACKNOWLEDGEMENTS

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I would like to thank Dominique Keller for her invaluable expertise in the field of reproductive physiology. Her patience and unselfish attitude were essential to the completion of this project. Whether I needed help running experiments or revising this thesis, she always made time for me.

I am grateful to the other members of the Reproductive Sciences Laboratory who supplied many valuable resources for this project. I would like to thank Lauri Willingham for her help with gel electrophoresis, Quinton Winger for help with statistical analysis, Carrie Hanna and Buck Williams for additional lab assistance, Taeyoung Shin, Gabriela Floures-Foxworth, and Jane Pryor for further reproductive expertise, and Sharon Jenkins and Linda Stolz for driving to Houston to obtain feline OHEs.

Other friends provided moral support without which this thesis would not have been completed. I would like to thank Brian Dear for staying late with me at the lab all of those nights, for computer help with PhotoShop, and for challenging me with questions about the significance of my work.

Finally, to those closest to me. I would like to thank my mother, grandmother, and Holly for being patient and supportive while I have been away at school.

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CHAPTER I

INTRODUCTION

In the world today, there are over twenty-five listed endangered feline species. Very little is known about these species; and if they become extinct, we may not have the chance to learn more about them. Tissues from nondomestic cats are not readily available. Therefore, studies are performed on tissues from domestic cats in the hope that the information gained can be transferred on to the rare species. Reproductive techniques developed for the domestic cat, such as *in vitro* maturation, *in vitro* fertilization, and embryo transfer, can potentially be applied to endangered feline species. Using these reproductive tools to propagate the endangered species, the diversity of the species may be maintained.

In the domestic cat, *in vitro* maturation (Johnston *et al*, 1989; Wood & Wildt, 1995), *in vitro* fertilization (Goodrowe *et al*, 1988; Pope *et al*, 1989, 1990, 1992; Johnston *et al*, 1990, 1991), and embryo transfer (Kraemer *et al*, 1979) have been performed. However, the techniques continue to be developed and perfected with more research. Also techniques such as chemical activation, which mimics the events at fertilization, and nuclear transfer, or cloning, are still under investigation in the domestic cat. All of these techniques may be useful in the preservation of endangered species.

Maturation indicates that the oocyte is competent to be fertilized. It is the

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This thesis follows the style and format of Molecular Development and Reproduction

requisite first step for *in vitro* fertilization, activation, and nuclear transfer. In nature, maturation usually occurs *in vivo*, but the technique is being perfected *in vitro* as well. During maturation, many different events and changes are occurring in the oocyte including changes in chromosome arrangement, changes in hormone levels, and changes in specific enzyme levels. Two such enzymes, Maturation Promoting Factor (MPF) and Mitogen Activated Protein Kinase (MAPK) are thought to be necessary for oocyte maturation and to be inactivated at fertilization or parthenogenic activation.

The first objective of this research was to perform controlled experiments to systematically compare the efficiency of three different maturation media *in vitro* on feline follicular oocytes. Three media differing in base medium, energy sources, and types of hormones were evaluated for their efficiency to mature feline oocytes to the metaphase II stage of meiosis. The second objective of this research was to perform a cell cycle analysis, evaluating the levels of two enzymes, MPF and MAPK, during meiosis and at activation in the feline oocyte.

CHAPTER II

REVIEW OF CURRENT LITERATURE

FEMALE FELINE REPRODUCTION

Between eight and ten months of age, the female cat reaches puberty (Jemmett & Evans, 1977). The first stage of the estrous cycle is proestrus, during which the female cat prepares for breeding and pregnancy. The estrus stage is the time of breeding. Estrus then occurs in 2-3 week intervals (Tsutsui & Stabenfeldt, 1993) and is usually 5-6 days long (Shille *et al*, 1979). Queens are seasonally polyestrus with the breeding season starting in January-February and ending in June-November (Stabenfeldt & Shille, 1977). Cats are reflex ovulators (Shille *et al*, 1979); therefore, ovulation occurs after mating or with artificial stimulus.

Following mating, there is a surge of Luteinizing Hormone (LH) which induces ovulation, although multiple copulations may be necessary for ovulation in the cat (Concannon *et al*, 1980; Wildt *et al*, 1980). If the queen does not mate or if ovulation does not occur, estrus will occur again in 2-3 weeks. If ovulation does occur, the corpora lutea form raising progesterone levels. The phase of progesterone dominance is defined as diestrus. In the nonpregnant cat, the luteal phase will last 30-50 days and estrus will occur again approximately one week after diestrus (Feldman & Nelson, 1996). If conception occurs, pregnancy lasts approximately 65 days (Lein & Concannon, 1983). The final stage of the estrous cycle is anestrus during which the cat does not have active ovarian function or exhibit sexual behavior.

IN VITRO MATURATION (IVM)

In vitro maturation involves the maturation of oocytes from the prophase, or germinal vesicle, stage of meiosis to the metaphase II stage of meiosis in an *in vitro* environment. Oocytes produced by *in vitro* maturation can potentially be used to replenish populations of endangered species, in genome banking projects, and for techniques such as *in vitro* fertilization and nuclear transfer. Also, the use of eggs matured *in vitro* from spayed animal tracts eliminates the need for laboratory animals to produce oocytes matured *in vivo*. Further research and development of IVM systems can help increase our understanding of the cytoplasmic and nuclear events occurring during meiosis as well as offer opportunities to preserve endangered wildlife.

In vitro maturation has been successfully performed in several mammalian species such as cats (Goodrowe *et al*, 1988; Pope *et al*, 1989, 1990, 1992; Johnston *et al*, 1990, 1991), cattle (Harper & Bracket, 1993), humans (Edwards, 1965), horses (Bezard & Palmer, 1992), mice (Eppig & O'Brien, 1996), swine (Tsafriri & Channing, 1975), and sheep (Moor & Trounson, 1977). However, maturation rates in the cat are lower than in other species (Wood *et al*, 1995). Several factors influence the ability of the feline follicular oocyte to be matured *in vitro* including storage intervals and maturation time (Goodrowe *et al*, 1991; Wolfe & Wildt, 1996), culture medium (Johnston *et al*, 1993, Luvoni & Oliva, 1993), protein and hormone supplementation (Johnston *et al*, 1991, 1993; Goodrowe *et al*, 1991; Luvoni & Oliva, 1993; Wood *et al*, 1995; Pope *et al*,

1997), and the overall morphological characteristics of the oocyte (Wood & Wildt, 1997; Pope *et al*, 1997).

Research has shown that the incidence of metaphase II in the oocyte peaks after 24 hours of culture, but maturation can continue up to 40 hours *in vitro* (Goodrowe *et al*, 1991). Johnston *et al* (1993) demonstrated that Eagle's Minimum Essential Medium was more efficient at maturing oocytes *in vitro* than Weymouth MB 753/1 Medium. Luvoni and Oliva's (1993) results indicated that Medium-199 supports feline oocyte maturation but at lower rates than other culture media. Previous results have also shown that the addition of FSH, LH, and estradiol increase the rate of feline oocyte maturation (Goodrowe *et al*, 1991; Wood *et al*, 1995). Pope *et al* (1997) concluded that oocytes supplemented with hCG had higher maturation percentages than those supplemented with FSH or eCG. The addition of BSA as a protein source has provided high rates of maturation *in vitro* (Wood *et al*, 1995) whereas the addition of FCS to the IVM culture system has been shown to be detrimental to the resumption of meiosis in feline oocytes (Luvoni and Oliva, 1993). Wood and Wildt (1997) and Pope *et al* (1997) concluded that oocytes with a uniform, dark cytoplasm and four or more layers of cumulus cells mature at a greater percentage than those with lesser amounts of cumulus cells or with lighter, fragmented cytoplasm. Thus, it can be seen that several factors contribute to the ability of the feline follicular oocyte to mature *in vitro*.

ACTIVATION

Oocyte activation occurs at fertilization and at parthenogenesis. Fertilization occurs when the male and female gametes fuse. The mature female gamete, or oocyte, is arrested at the metaphase II stage of meiosis and typically will die without activation. Activation includes the morphological and physiological events that trigger the oocyte's release from metaphase II arrest, resumption of meiosis, and the initiation of mitotic divisions.

The fusion of the sperm with the egg during fertilization causes intracellular calcium to be released in the oocyte's cytoplasm in a series of waves that can last for several hours (Miyazaki *et al*, 1993). The calcium oscillations commence oocyte activation and last until the time of pronucleus formation (Miyazaki *et al*, 1993; Swann & Ozil, 1994; Jones, 1998). During activation, the zona pellucida is modified to block polyspermy, the second polar body is extruded, and pronucleus formation occurs (Xu *et al*, 1994; Wu *et al*, 1998; Ben-Yosef & Shalgi, 1998). Studies in several animal species show that after activation, the activity of the cell cycle regulatory enzymes, MPF and MAPK, also decreases. The increase in intracellular Ca^{2+} results in degradation of cyclin B, a component of MPF, leading to inactivation of MPF. The inactivation of MPF allows the activated oocyte to be released from meiotic arrest. MAPK levels also drop; high levels of MAPK have been reported to be incompatible with pronucleus formation (Moos *et al*, 1996). Once activation occurs, progression to the first mitotic divisions proceeds, and embryonic development can begin.

Parthenogenesis is the production of an embryo from the female gamete without contribution from the male gamete. Parthenogenic activation by external chemical and physical activators has been induced in mammalian oocytes from many different species *in vitro*. The oocyte is exposed to stimuli that mimic the effects of fertilization and mitotic divisions proceed. Calcium ionophores (Hagemann *et al*, 1995), electrical pulses (Stice *et al*, 1994), calcium injection (Swann & Whitaker, 1986), and ethanol (Tateno & Kamiguchi, 1997) have been used to activate oocytes of various mammalian species. These artificial activators also trigger calcium release in the oocyte; but instead of calcium oscillations, usually only one calcium spike occurs (Kline, 1996). Although these conditions are not ideal, parthenogenesis can occur.

CELL CYCLE CONTROL WITH MPF AND MAPK

Maturation Promoting Factor (MPF) is a histone H1 kinase composed of cyclin B and $p34^{cdc2}$ kinase. The activity of MPF in bovine, mouse, and frog eggs has been shown to rise and fall in a cyclic manner (Liu *et al*, 1998, 1999; Taieb *et al*, 1997; Dale *et al*, 1999). The levels of MPF are low at the germinal vesicle (GV) stage of meiosis, rise during the germinal vesicle breakdown (GVBD) and metaphase I (MI) stages, drop during anaphase I, and peak again at metaphase II (MII). At fertilization or parthenogenic activation, the activity of MPF decreases, possibly due to the calcium spikes initiating the degradation of the cyclin B portion of MPF (Liu *et al*, 1998). At fertilization, MPF is inactivated and not regenerated once the cyclin B is degraded. With parthenogenic activation, it is possible, under certain conditions, for the MPF to

regenerate once the activating influence is withdrawn (Liu & Yang, 1999). With incomplete activation, the oocyte can become arrested in metaphase III, incapable of further development (Liu *et al*, 1998). A second agent must be applied after the calcium elevating agent in order to prevent the reactivation of MPF. Cycloheximide, a protein synthesis inhibitor, prevents resynthesis of cyclin (Presicce and Yang, 1994). 6-DMAP, a protein kinase inhibitor, will also keep MPF from regenerating (Liu *et al*, 1998). With complete activation, the levels of MPF will decrease until the point of the first mitotic division (Presicce and Yang, 1994). Then, the activity of MPF continues to rise and fall in a cyclic manner with each division.

Mitogen Activated Protein Kinase (MAPK) is another enzyme involved in oocyte maturation. During maturation, it is thought to help maintain chromosome condensation and help form the meiotic spindle (Choi *et al*, 1996). MAPK activity rises and falls in the same manner as MPF activity; however, MAPK activity lags behind that of MPF. After activation, MAPK levels decrease for pronucleus development to occur (Liu & Yang, 1999).

CHAPTER III

METHODS AND MATERIALS

OOCYTE COLLECTION

Domestic cat ovaries were collected from ovariohysterectomy material provided by local veterinary clinics and held in Physiological Saline Solution (PSS) until processing. Oocytes were recovered from excised ovaries by mincing with a scaple blade at room temperature in Tyrode's lactate Hepes buffered medium (Biowhittaker, Walkersville, MD) supplemented with 3 mg/ml BSA (TL Hepes). Eggs were washed twice in TL Hepes before selection, grading, and placement in treatment culture.

CLASSIFICATION OF OOCYTES

Oocytes were selected and graded as good, fair, and poor based on defined morphological criteria (Wood & Wildt, 1997; Pope *et al*, 1997). Good oocytes had 4 or more layers of cumulus cells and a dark, uniform cytoplasm. Fair oocytes had 2-4 layers of cumulus cells and a less darkly pigmented cytoplasm. Poor oocytes had 1 or less layers of cumulus cells and a pale or unevenly granulated cytoplasm.

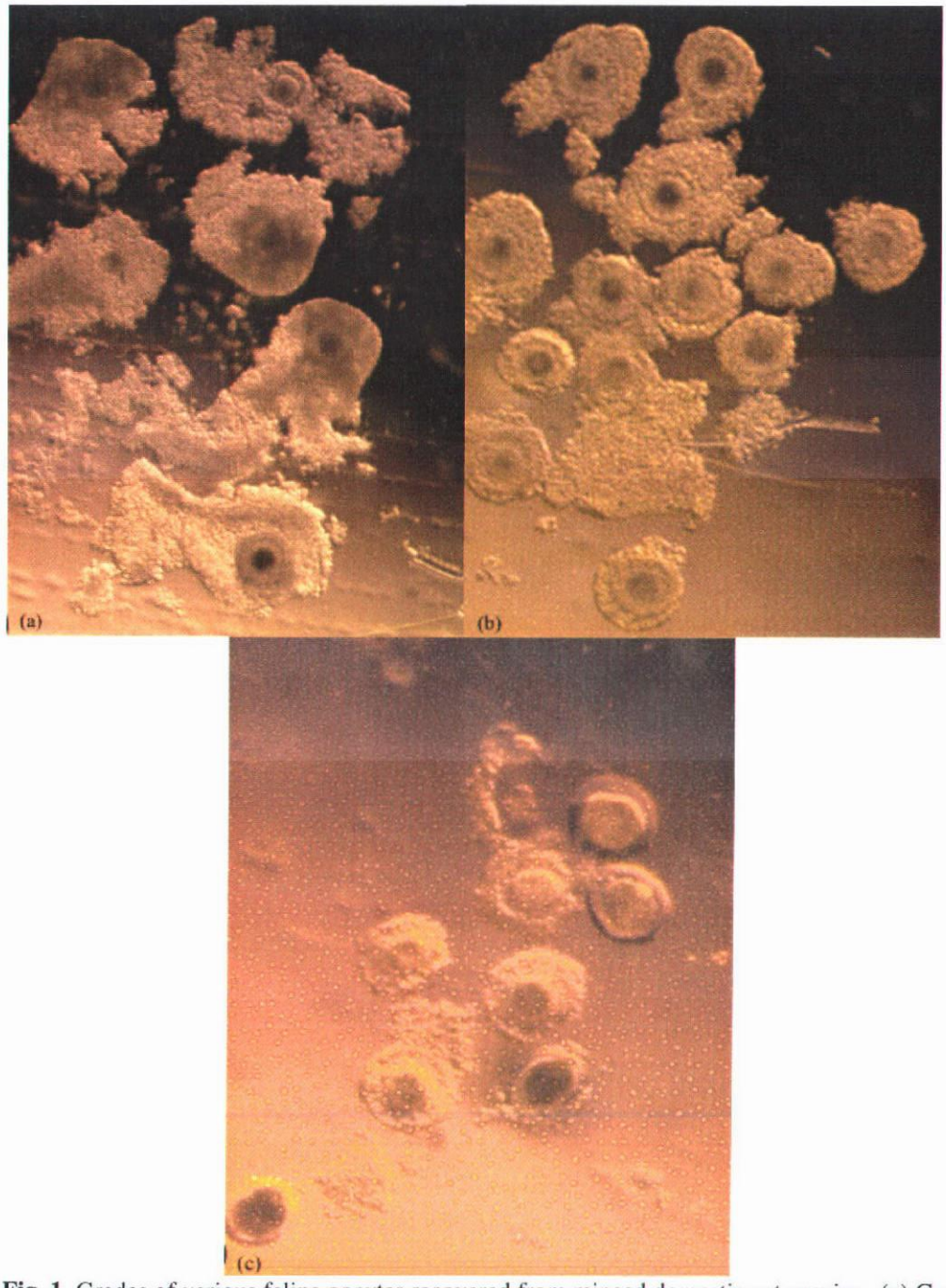

Fig. 1. Grades of various feline oocytes recovered from minced domestic cat ovaries. (a) Good: oocytes have a dark, uniform cytoplasm with 4 or more layers of tightly compacted cumulus cells. (b) Fair: oocytes have 2-4 layers of cumulus cells and a less darkly pigmented cytoplasm as compared to good oocytes. (c) Poor: oocytes have mosaic transparent or fragmented cytoplasm and 1 or less layers of cumulus cells, some nearly denuded.

CULTURE TREATMENTS FOR IVM

To compare the efficiency of maturation medium on feline follicular oocytes *in vitro*, three media differing in base medium, energy sources, and hormones were tested. Oocytes of each grade--good, fair, and poor (Fig.1)—were randomly divided into 50 µl drops of one of the three equilibrated media, covered with mineral oil, and incubated for 24 hours at 38.5°C in 5% CO_2 humidified in air. Fifteen to twenty oocytes were placed in each drop. All chemicals were purchased from Sigma (St. Louis, MO) except as noted. The first treatment medium (W1) contained Eagle's Minimum Essential Medium, 0.026g/l pyruvate, 0.292g/l L-glutamine, 0.4% (w/v) BSA, 100 iu/ml penicillin (Gibco, Grand Island, NY), 100 iu/ml streptomycin (Gibco), 1 µg/ml LH (Sioux Biochemicals, Sioux City, IA), 1 µg/ml FSH (Sioux Biochemicals), and 1 µg/ml estradiol (Wood *et al*, 1995). The second treatment medium (W2) contained the same base medium and energy sources as the first treatment with 2 µg/ml LH, 2 µg/ml FSH, and 2 µg/ml estradiol . The third treatment medium (CREW) contained Medium-199 (Gibco BRL, Grand Island, NY) supplemented with 0.36 mM pyruvate, 0.78 mM calcium lactate, 0.063 g/l penicillin, 0.050 g/l streptomycin, 3.0 g/l BSA Fraction V, 1 µg/ml FSH, 1 iu/ml hCG (Sigma) (Cincinnati Zoo). Controls were run using the same base maturation media without the hormones. Figure 2 shows feline oocytes before and after IVM culture.

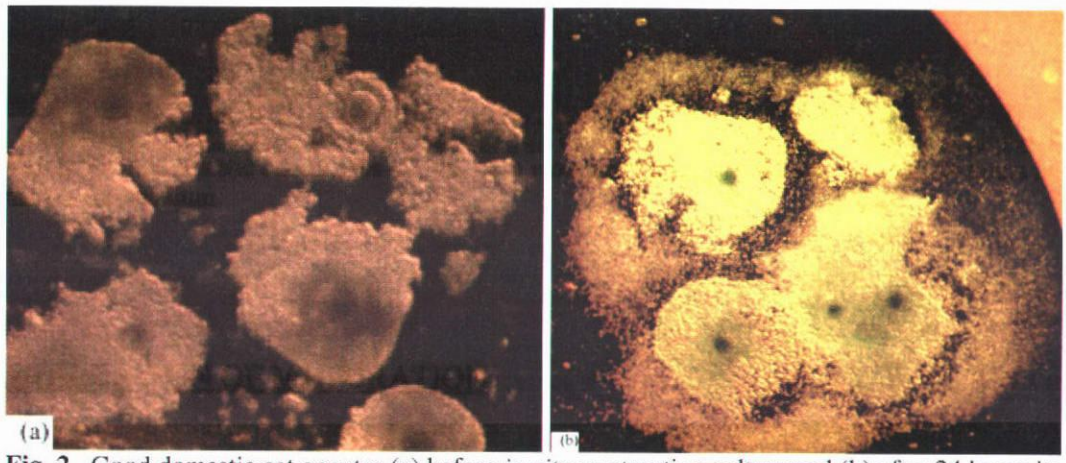

Fig. 2. Good domestic cat oocytes (a) before *in vitro* maturation culture and (b) after 24 hours in maturation medium

EVALUATION OF MATURATION

After IVM culture for 24 hours, the meiotic status of all oocytes was evaluated. First, eggs were vortexed for 3 minutes to remove expanded cumulus cells. Oocytes were fixed in 3.7% paraformaldehyde with Triton X-100 , washed with DPBS-PVP, and stained with Hoechst 33342 in glycerol. Under UV light, the meiotic stage of each oocyte was assessed under a microscope (Fig. 3). Oocytes reaching metaphase II (MII) were considered mature. All oocytes in the germinal vesicle, germinal vesicle breakdown, metaphase I, and anaphase I stages of meiosis as well as oocytes with unidentifiable nuclear material were considered immature. The medium concluded to be the most efficient was used for *in vitro* maturation in all further experiments. Differences in means amongst treatment groups was determined by Chi^2 analysis.

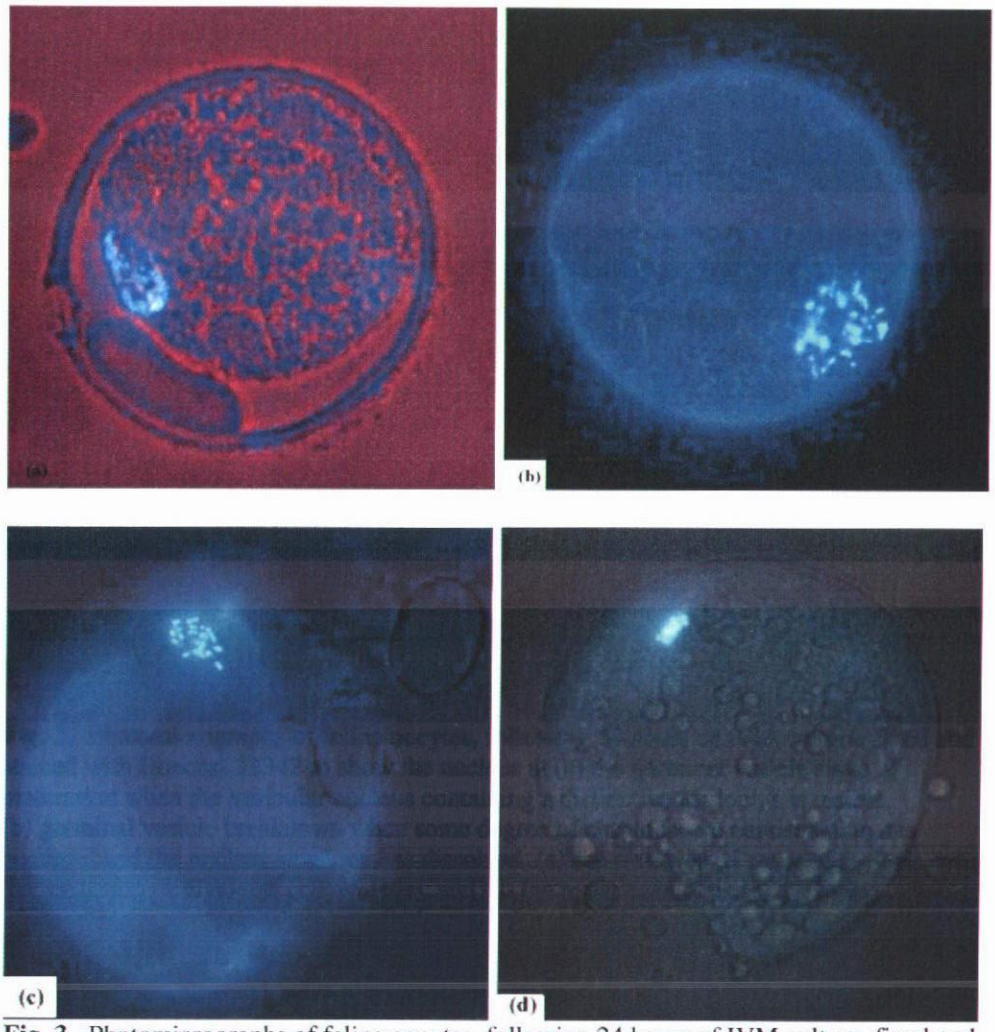

Fig. 3. Photomicrographs of feline oocytes, following 24 hours of IVM culture, fixed and stained with Hoechst 33342 to show the nucleus at (a) the germinal vesicle stage of maturation when the vesicular nucleus containing a distinctive nucleolus is visible, (b) germinal vesicle breakdown when some degree of chromosome condensation has occurred and the nuclear envelope has dissolved, (c) the metaphase I stage of meiosis, when the formation of bivalents is completed, and (d) the metaphase II stage of meiosis, when one group of chromosomes is lined up on the metaphase plate, allowing individual chromosome identification, and the other group of chromosomes is tightly packed into the first polar body.

CHEMICAL ACTIVATION

After culture in maturation medium for 24 hours, cumulus cells were removed from oocytes by vortexing for 3 minutes. Once the oocytes were denuded, those with a polar body were selected for activation treatment. Half of the oocytes with polar bodies were activated for 4 minutes in 5 mM ionomycin (Calbiochem, San Diego, CA) followed by inactivation in TL Hepes supplemented with 30 mg/ml BSA (4 min). They were then incubated in cycloheximide for 5 hours followed by culture for 15 hours in Medium-199 with 10% FCS, 100 iu/ml penicllin, and 100 iu/ml streptomycin. The other half of the oocytes with polar bodies were treated as controls and incubated for 20 hours in Medium-199 supplemented with 10% FCS, 100 iu/ml penicillin, and 100 iu/ml streptomycin. The results of this experiment were used to evaluate the effectiveness of this activation treatment on feline oocytes and to see if this treatment would be useful for the cell cycle analysis.

EVALUATION OF CHEMICAL ACTIVATION

After activation and culture for 20 hours, the activation of the oocytes was assessed with the same fixation and staining procedure as for maturation. Successful activation was evidenced by the formation of at least one pronucleus (Fig. 4). Mitotic cell division was also an indication of activation. Degenerating oocytes or those still arrested at metaphase II were not considered activated.

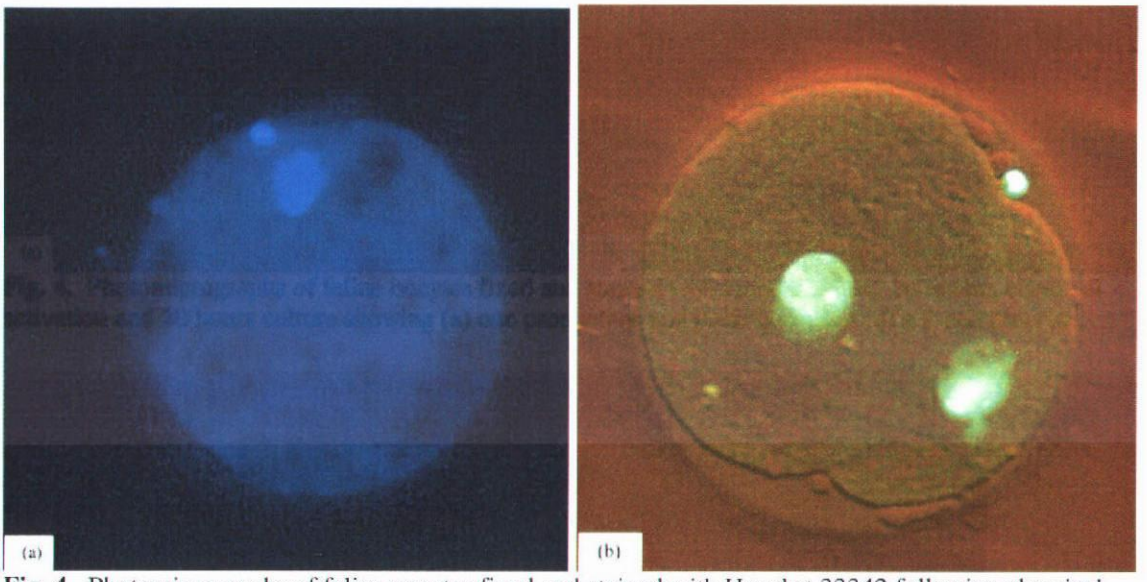

Fig. 4. Photomicrographs of feline oocytes fixed and stained with Hoechst 33342 following chemical activation and 20 hours culture showing (a) one pronucleus and polar body and (b) a 2-cell embryo.

CELL CYCLE ANALYSIS

MPF activity and MAPK activity were measured by running a SDS-PAGE gel assay using histone H1 and myelin basic protein (MBP) as substrates (Fissore *et al*, 1996). Five oocytes from each time point to be studied—germinal vesicle stage, metaphase I, metaphase II, and activated/control oocytes from time points at activation, 10 hours post activation, and 20 hours post activation were collected. The control oocytes did not receive the activation treatment. Each collection was stored in 5 µl of an H1 kinase buffer solution containing 10µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 500 nM protein kinase A inhibitor, 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl, and 1 mM dithiothreitol (Collas *et al*, 1993). Eggs were lysed, releasing their contents, by repeated freezing and thawing and then stored at -80°C until the assay was performed. Kinase reactions were started by adding 5 µl of a solution

containing 2 mg/ml histone H1, 1 mg/ml MBP, 0.7 mM ATP (Amersham-Pharmacia Biotech), and 50 μCi of [γ-^{32}P]ATP (Amersham-Pharmacia Biotech) to the 5 μl egg samples. The reaction was carried out for 30 minutes at 35°C and ended by adding 5 μl of SDS sample buffer (Laemmli, 1970). Samples were boiled for 3 minutes and loaded into precast Ready Gels (Biorad, Hercules, CA). Phosphorylation of histone H1 and MBP was visualized by autoradiography using Bio Plus intensifying screens (CBS Scientific, Del Mar, CA). Following gel exposure for 3-5 hours, band intensities of the autoradiograms were determined by densitometry using the Alphaimager. Kinase activity for the oocytes during meiosis is shown relative to the kinase activity of GV oocytes, and the kinase activity for oocytes during activation is shown relative to the kinase activity of MII eggs. Data are presented as means ± SEM.

DATA ANALYSIS

Chi square analysis was used to evaluate the *in vitro* maturation and oocyte activation experiments. Comparison between different stages of meiosis and during activation, with regard to MPF and MAPK activity, was carried out using a student's t-test. Differences at $p<0.05$ were considered significant. All of the experiements were replicated at least two times.

CHAPTER IV

RESULTS

IN VITRO MATURATION

From 100 ovaries, 359 oocytes were selected as good, 821 as fair, and 403 as poor. Results from the IVM experiments are shown in Table 1.

TABLE 1. Maturation of domestic cat oocytes cultured *in vitro*

Treatment[a]	No. of Eggs			No. of Eggs at Metaphase II (%)		
	Good	*Fair*	*Poor*	*Good*	*Fair*	*Poor*
W1	124	229	60	32 (26)	41(18)	5(8)
W2	126	317	151	55(44)	69(22)	16(11)
W1,2 Control	19	61	44	0 (0)	0 (0)	0 (0)
CREW	64	172	114	14(22)	19(11)	4(3.5)
CREW Control	26	42	34	0 (0)	0 (0)	0 (0)

[a]Oocytes were cultured in 50 µl drops with 15-20 oocytes per drop under mineral oil at 38.5°C in 5% CO_2 in humidified air.

As shown in Figure 5, W2 (treatment 2) demonstrates the greatest efficiency of maturing good quality feline follicular oocytes *in vitro* of the 3 treatments tested. Figures 6 and 7 show the percent maturation to metaphase II of the fair and poor oocytes.

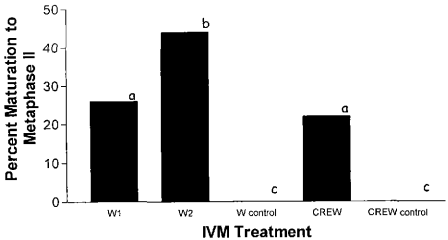

Fig. 5. Percentages of good domestic cat oocytes reaching metaphase II after maturation in each of three maturation media. Columns with different superscripts show significant difference ($p<0.05$).

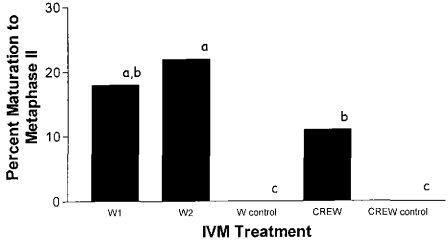

Fig. 6. Percentages of fair domestic cat oocytes reaching metaphase II after maturation in each of three maturation media. Columns with different superscripts show significant difference ($p<0.05$).

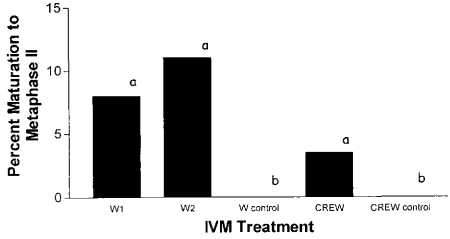

Fig. 7. Percentages of poor domestic cat oocytes reaching metaphase II after maturation in each of three maturation media. Columns with different superscripts show significant difference ($p<0.05$).

CHEMICAL ACTIVATION

Twenty oocytes were activated with the ionomycin/cycloheximide activation treatment, and twenty oocytes were used as controls in order to test the ability of this treatment to activate mature feline oocytes (Table 2). Figure 8 shows that the treatment was successful in inducing oocyte activation resulting in pronucleus development after culture for 20 hours. Some cells had reached the 2-cell stage of development when fixed and stained.

TABLE 2. Activation of mature domestic cat oocytes with ionomycin/cycloheximide

Treatment	No. of Eggs	No. of Eggs with 1 Pronucleus(%)	No. of Eggs Cleaved to 2-cells(%)
Activated[a]	20	8(40)	10(50)
Unactivated Controls[b]	20	1(5)	1(5)

[a]Mature oocytes were treated with ionomyic (4min), inactivation medium (4min), and cycloheximide (5hrs). They were then cultured for 15 hrs in M-199 supplemented with 10% FCS and 1% pen/strep.
[b]Mature oocytes were cultured for 20 hrs. in M-199 supplemented with 10% FCS and 1% pen/strep.

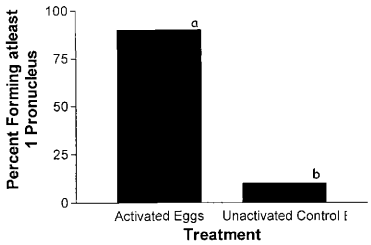

Fig. 8. Percentage of mature domestic cat oocytes forming at least one pronucleus after activation treatment with ionomycin/cycloheximide or control treatment. Columns with different superscripts indicate significant difference ($p<0.05$).

CELL CYCLE ANALYSIS

In this experiment, the activity of MPF and MAPK were tested by quantifying bands from a separating gel. The activity of MPF and MAPK changed during meiosis as evidenced by the radioactivity of the bands in the gel (Fig. 9). The levels of MPF and MAPK during meiosis are summarized in Figures 10 and 11. MPF and MAPK activities are higher at both the metaphase I and metaphase II stages of meiosis versus the germinal vesicle stage.

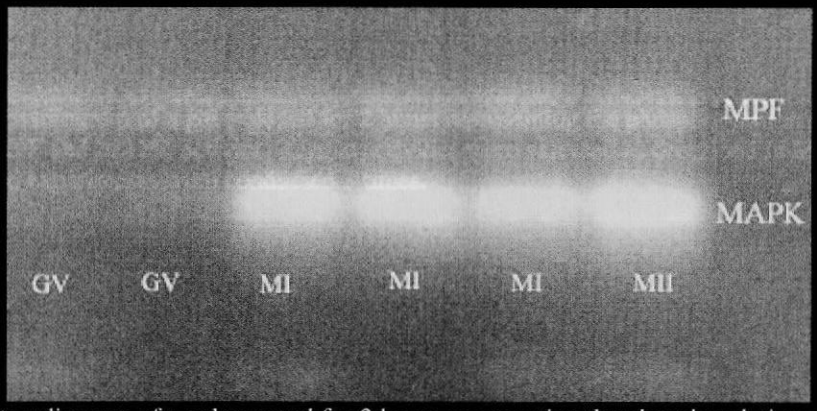

Fig. 9. Autoradiogram of a gel exposed for 2 hours representing the phosphorylation of H1 and MPB, and hence MPF and MAPK activity. Kinase activities were assessed as described in Materials and Methods, and experiments were conducted 3 times.

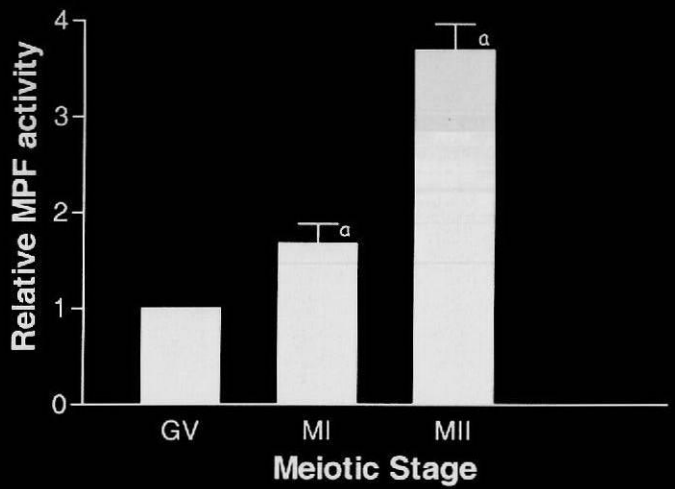

Fig. 10. Quantification of the change in MPF activity during meiosis in the domestic cat. Data are presented as means ± SEM, and kinase activities are expressed relative to the kinase activity in GV eggs, which were arbitrarily given the value of 1. Values with a superscript are significantly different from those in the GV stage ($p<0.05$).

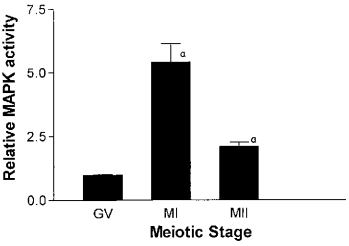

Fig. 11. Quantification of the change in MAPK activity during meiosis in the domestic cat. . Data are presented as means ± SEM, and kinase activities are expressed relative to the kinase activity in GV eggs, which were arbitrarily given the value of 1. Values with a superscript are significantly different from those in the GV stage ($p<0.05$).

The activities of MPF and MAPK during activation were also noted by the change in radioactivity of the gel bands (Fig. 12). Figures 13 and 14 show MPF and MAPK activity at activation, 10 hours post activation, and 20 hours post activation along with controls. Both enzymes showed high levels of activity at time=0. Ten hours after the activation stimulus, activity of both enzymes decreased abruptly. The MAPK levels decreased even further up to 20 hours after the activation stimulus, but the MPF activity increased again by this time point.

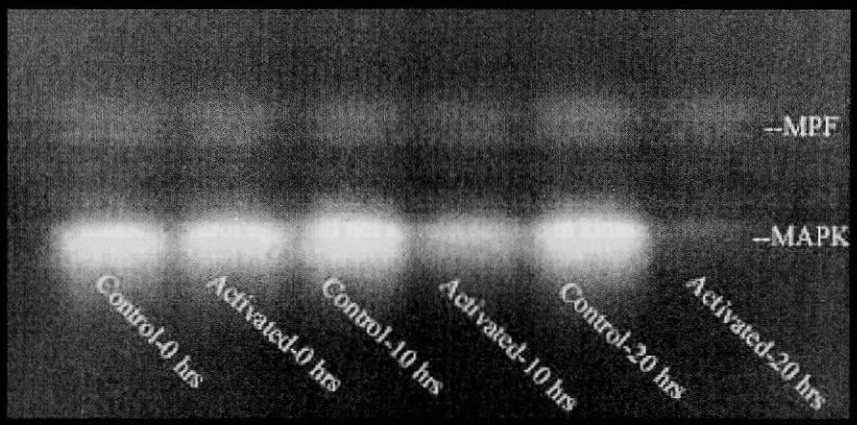

Fig. 12. MPF and MAPK activity decreases after chemical activation of feline oocytes. Domestic cat oocytes (5 per lane) were either activated or not activated and cultured for 0, 10 , and 20 hours post activation. Kinase activities were assessed as described in Materials and Methods, and experiments were conducted three times. Control eggs did not show a decline in kinase activity. Conversely, activated eggs did show a decline in MPF and MAPK activity, but at 20 hours, the activity of MPF rose again.

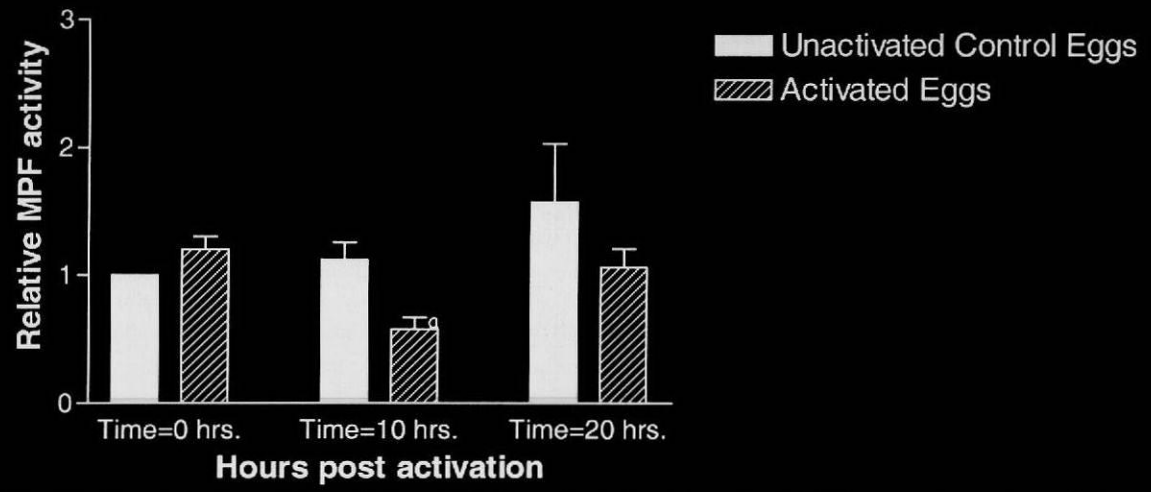

Fig. 13. Quantification of the activity of MPF in activated and unactivated feline oocytes at three different time points. Feline oocytes were either treated with ionomycin/cycloheximide followed by culture in M-199 or were simply cultured in M-199. Data are presented as means ± SEM, and kinase activities are expressed relative to the kinase activity in MII eggs (control eggs at time=0 hrs.), which were arbitrarily given the value of 1. Values with a subscript are significantly different than those in the control group ($p<0.05$). Experiments were repeated three times.

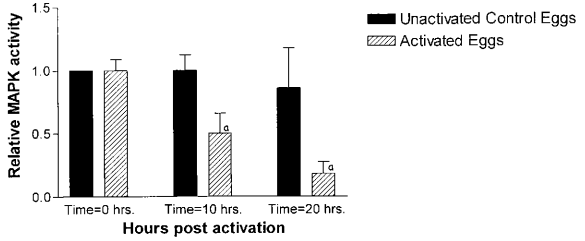

Fig. 14. Quantification of the activity of MAPK in activated and unactivated feline oocytes at three different time points. Feline oocytes were either treated with ionomycin/cycloheximide followed by culture in M-199 or were simply cultured in M-199. Data are presented as means ± SEM, and kinase activities are expressed relative to the kinase activity in MII eggs (control eggs at time=0 hrs.), which were arbitrarily given the value of 1. Values with a subscript are significantly different than those in the control group ($p<0.05$). Experiments were repeated three times.

CHAPTER V

CONCLUSION

IN VITRO MATURATION

The results of this study show that the W2 *in vitro* maturation medium was the most efficient of the three media tested. While all three culture treatments in this experiment supported the resumption of meiosis and its completion to the second meiotic metaphase, in the case of good quality oocytes, increasing the concentration of hormones seems to most positively affect the efficiency of the medium. When techniques such as nuclear transfer are used good oocytes are most likely to be used. Therefore, the most efficient way of maturing good oocytes is of greatest concern.

Maturing oocytes *in vitro* has many applications. The mature eggs can be used in reproductive techniques, such as *in vitro* fertilization, which can be applied to the conservation of endangered species. Maturing and fertilizing immature oocytes *in vitro* provides a method for rescuing genetic material from exotic and endangered feline species. Using oocytes from ovaries of spayed felines also lowers the number of laboratory animals that are needed to study these techniques. Nuclear transfer, which relies on mature oocytes, is also an important technique because it can potentially be used to study environmental versus genetic components of disease. Observing oocytes throughout maturation also helps one better understand events taking place in the oocyte during meiosis.

ACTIVATION

Biotechniques, such as nuclear transfer and sperm injection, require the activation of the recipient oocytes for meiosis to continue and result in embryonic development. Artificial activation can also be used to observe the events occurring in the oocyte during activation and early cleavage. In this study, one chemical activation treatment was tested in feline oocytes for effectiveness. At this time, there are no published reports on feline activation protocols, so ionomycin/cycloheximide was tested due to its success in the oocytes of several other mammalian species.

The results indicate that the ionomycin/cycloheximide treatment activated feline oocytes, resulting in pronucleus development and cleavage. At 20 hours post activation, a higher rate of 2-cell embryos was observed in the feline oocytes compared to observed rates in some other species such as bovine. This effect may be due to the activation treatment, timing of the feline cell cycle, or other factors. Further investigation is needed to elucidate the actual cause.

Further study of activation treatments for feline oocytes is also necessary. A comparison of the efficiency of activation treatments will be beneficial for the reproductive techniques that rely on activation. Once the most effective activation treatment for feline oocytes is perfected, the efficiencies of these other techniques may increase as well.

CELL CYCLE ANALYSIS

The activities of MPF and MAPK during meiosis and at activation in the feline oocyte, as determined by this study, appear to be similar to the activities in other species studied, but a more in depth analysis is needed. This study shows that the activities of MPF and MAPK at both metaphase I and metaphase II are higher than at the germinal vesicle stage of meiosis, which concurs with previous studies. However, the levels of these enzymes also should be studied during germinal vesicle breakdown and at anaphase I in order to obtain a more complete picture. In other species, these are also critical stages for MPF and MAPK activity.

The activities of MPF and MAPK during activation of feline oocytes also agrees with previous studies. The levels of both these enzymes decrease after the activation stimulus. In this study, the levels of MPF had risen again at 20 hours post activation. This rise in activity is possibly due to the earlier observation that the oocytes had already started to cleave into 2-cell embryos at the 20 hour time point and thus had entered the mitotic cell cycle. The levels of MPF and MAPK are also thought to rise and fall with each mitotic division, with MAPK lagging behind MPF. In the bovine, a sharp rise in oocyte MPF was observed during the first mitotic cleavage, whereas MAPK activity stayed low (Liu *et* al, 1998). Therefore, it is possible for MPF activity to have risen again due to the first mitotic division while MAPK activity remained low. In order to more clearly evaluate the events taking place, a similar study evaluating more frequent time points is necessary. Cytoskeletal events and chromatin behavior during meiosis and activation as well as the components of MPF and MAPK are also important to

investigate in order to better understand the cell cycle and to understand what occurs during meiosis and mitosis.

The cell cycle analysis of MPF and MAPK is important for understanding the events taking place during meiosis and at activation in the feline oocyte on a biochemical level. It allows us to determine whether or not the cat's oocyte cell cycle is similar to that of other species and if similar activities are occurring. It also further validates the maturation and activation treatments used for the studies.

CONCLUSION

Research and development of feline reproductive techniques has many potential applications, from endangered species conservation to the study of diseases. The research presented in this study can be used to further understand the events occurring in the feline cell cycle from meiosis through activation. This study shows that increasing hormone levels in maturation culture medium increases the rate of feline oocyte maturation *in vitro*. The cell cycle analysis portion of this study shows that MPF and MAPK patterns in the feline appear to be similar to that of other species. However, more research is needed at the biochemical level to ensure proper use of techniques such as *in vitro* fertilization, nuclear transfer, and sperm injection.

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