ELIZABETH VIRGINIA DEW
In Vitro Maturation (IVM) of the Canine Oocyte.
(Under the direction of RICHARD A. FAYRER-HOSKEN)

This research examines culture techniques to improve nuclear maturation of canine oocytes in vitro. The primary objective of these experiments was to test individual; 1) estradiol or progesterone, 2) FSH, 3) eCG, 4) LH, 5) L-cysteine, 6) arachidonic acid, effects and varying lengths of culture on IVM of canine oocytes. The effects of the individual treatment media was compared to control media containing no supplement.

For all experiments, the oocytes were collected from bitches undergoing ovariohysterectomies. Nuclear maturation assessment used Hoechst 33342 fluorescent stain. In total, 8,536 oocytes were examined in these experiments. A significant ($p<0.05$) improvement for maturation rates of oocytes to metaphase II was obtained when they were cultured for 96 hours with rFSH. All other media supplements tested did not significantly increase maturation of canine oocytes. For future canine oocyte nuclear maturation, rFSH should be used, probably, in conjunction with other supplements.

INDEX WORDS: canine, in vitro maturation, oocyte

IN VITRO MATURATION OF THE CANINE OOCYTE

by

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DEDICATION

These manuscripts are dedicated to my family and my fiancée, without their constant love and support my graduate education would not have been possible.

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I would like to thank the many local veterinary clinics whose clinicians were kind enough to save canine ovaries for my project. My committee members Doctors Dookah and Rampacek along with my major professor Dr. Fayrer-Hosken have provided much needed guidance along the way. Also, much thanks goes to John Cartee and his expert ovary transport skills.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Over the past 50 years, scientists have made great progress in the field of assisted reproduction technologies (ART) for a variety of species. Bovine in vitro maturation (IVM) and in vitro fertilization (IVF) techniques have served well as a model for developing similar systems for other species including human, goat, and feline. Live births of IVF offspring are frequent in veterinary and human medicine, Application of IVM/IVF technologies in the dog, however, have beenextremly problematic. Techniques successfully developed for other species have yet to produce IVF puppies. Reports of in vitro produced canine embryos are rare. Successful transfer of canine embryos which were in vivo fertilized in one bitch, and transferred to the uterus of an estrus synchronized surrogate bitch was achieved in 1989 resulting in the birth of 3 live puppies (17). To date, only one pregnancy from IVF has been reported in the dog (7). This pregnancy did not go to term. This implies that once sucessful and repeatable techniques have been developed for IVM and IVF of canine oocytes, it will be possible to use embryo transfer to an estrus synchronized bitch to obtain live births and offer this technique as a possible clinical treatment for infertility.

A possible explanation of the difficulties experienced with IVM of canine oocytes may be attributed to the unusual dog reproductive cycle and physiology (1). In contrast to most species, the dog ovulates an immature or primary oocyte at the germinal vesicle stage of nuclear maturation (1). Upon ovulation in the bitch,which occurs two days after the LH surge. The primary oocyte having immature nuclear components, leaves the ovarian

follicle and enters the bursa and canine oviduct. While in the oviduct, the oocyte undergoes nuclear maturation to metaphase II up to two days after ovulation. The oviductal oocytes are then fertilized (1,15). Additionally, the morphology of the canine oocyte is markedly different from that of many other species. Most notable is the dense "lipid-like" material within the ooplasm (1). This lipid-like material has made staining and nuclear assessment of the canine oocyte especially challenging.

Development of IVM/IVF techniques providing successful production of viable canine embryos for embryo transfer, would be applicable to many facets of veterinary medicine both clinical and research. Overcoming the current difficulties associated with the application of IVM/IVF in the dog will provide scientists and veterinarians a greater understanding of canine reproductive physiology. Further, improved IVM/ IVF techniques in the dog will lead to the application of ART to conserve valuable genetic resources in a variety of endangered canids and other species.

CANINE OOCYTE DEVELOPMENT, MATURATION AND OVULATION

The mammalian female haploid germ cell is termed an oocyte. Oocytes first develop in female mammals prior to birth. Haploid germ cells are derived from primordial germ cells which are first visible in the epithelium of the dorsal endoderm of the embryonic yolk sac, near the allantois (1). Primordial germ cells migrate from the embryonic yolk sac, proceeding along the embryo's hindgut, to reach their final destination in the ventral embryonic kidney (1). Migration of the primordial germ cells occurs by an amoeboid like movement enhanced by chemotactic factors released by the presumptive gonads that attract primordial germ cells to this region (1). Throughout their migration, primordial germ cells are undergoing mitosis and, therefore, increasing in number (1).

Upon completing migration, germ cells reach an area referred to as the genital ridge, the site of gonad development (1). Within the genital ridge, germ cells are termed oogonia

in the XX (female) embryo and prospermatogonia in the XY (male) embryo (1). In the human embryonic ovary, oogonia begin the transformation to oocytes during the third month of gestation when meiotic prophase commences (1). By the time of birth, all germ cells in the human female infant have become oocytes (1). A wide variation is seen among females of various species regarding the stage of development for commencement of meiosis, although for most species, meiosis has been reported to begin at a point prior to birth (1).

In contrast to males, females are born with a finite number of germ cells. Once mitotic division has ceased, the oogonium is termed an oocyte and meiotic division begins. Meiosis of the initially diploid germ cell, referred to as a primary oocyte, is composed of two stages, the first of which results in a haploid cell. Exchange of genetic information between chromosomes can occur during the first division of meiosis in a processes referred to as crossing over. Having undergone the first meiotic division a primary oocyte is now referred to as a secondary oocytes which is haploid, containing 23 chromosomes (1). During the second meiotic division, each pair of chromatids is separated resulting in an egg with single, unpaired chromosomes (1).

Meiotic cell division is divided into four different phases: prophase, metaphase, anaphase and telophase (1). Interphase is used to describe the time between successive cell cycles; DNA replication occurs during interphase (1). Prophase of the first meiotic division is divided into five stages for discussion: leptotene, zygotene, pachytene, diplotene and diakinesis. Diakinesis is also referred to as germinal vesicle stage (1). The long prophase of the first meiotic division contains two periods of time when development is arrested, the dictyate stage and either metaphase I or II (1).

For most mammalian species, while vesicular and growing follicles are evident in the female ovary within the first few days of birth, meiotic prophase ceases at the diplotene stage within a week of birth. During puberty, initiation of the cyclic secretion of reproductive hormones causes follicular growth to continue allowing resumption of meiosis to occur as the result of preovulatory changes in the Graafian follicle, After initiation of puberty,

during each estrus cycle, initial follicular growth begins with an increase in the size of the oocyte, the granulosa cells becoming more cuboidal, along with formation of the zona pellucida (1). As the anterior pituitary hormone, follicle stimulating hormone (FSH), induces follicles to grow and mature. The number of granulosa cells surrounding the growing follicles increases as does the amount of fluid that accumulates between the granulosa cells forming a cavity termed the antrum of the follicle (1). Follicles containing a fluid filled antrum are referred to as Graafian follicles (1). The final growth of Graafian follicles is dependent not only on FSH but also the presence of LH. Final preovulatory maturation of Graafian follicles and oocytes for most mammals occurs during a change in the ratio of FSH and LH. This time shortly before ovulation is particularly noted by an increase in circulating LH. For most mammalian species, during the LH surge an increase in antrum fluid in the Graafian follicle occurs simultaneously with the resumption of meiosis within the oocytes (1). Ultimately ovulation commences, after which, the ruptured follicle becomes the corpus hemorrhegica which becomes the corpus luteum. This produces the steroid hormone progesterone, crucial for maintaining pregnancy (1). Due to a finite number of germ cells at birth in the female, the number of oocytes a female has remaining in the ovary will continually decline due to ovulation as the animal ages until a halt of reproductive function in some species as seen in humans during menopause (1).

For most mammals, the egg, the primary oocyte present at the beginning of preovulatory maturation and has reached metaphase of the second meiotic division (MII) prior to ovulation (1). This is not true for the fox and dog which ovulate a primary oocytes containing an intact germinal vesicle or considered to be in a stage shortly after the start of the first meiotic division (MI) (1). Oocytes of the dog and fox complete the first meiotic division and extrusion of the first polar body after ovulation, once the oocyte is in the oviduct (1).

OOCYTE MORPHOLOGY

Morphology of the canine oocyte includes a cytoplasm bound by a plasma membrane containing the nucleus, or condensed chromosomes, and various organelles. Mitochondria, endoplasmic reticulum and the Golgi apparatus comprise the majority of organelles of the oocyte (1). While the oocyte is within the ovary, the Golgi apparatus plays an important role in yolk synthesis and formation of cortical granules which are a key factor during fertilization in preventing polyspermy (1). Spindles which developed from microtubules, form during meiosis and mitosis to aid in the alignment of chromosomes (1). Cumulus cells are thought to play a vital role in providing the oocyte with nourishment while in the follicle (1). These cells also provide a surface for fimbrial cilia to work against while the egg is being transported within the oviduct (1).

Morophologic changes that occur within the canine oocyte during different stages of the estrus cycle may affect the ability of the canine oocyte to communicate with cumulus cells involving meiotic competence (18). In mammalian ovaries, gap junctions exist between the somatic compartment of the follicle and the oocyte. Philips and Dekel described these gap junctions as being involved in the regulation of oocyte meiotic differentiation and maturation leading to the acquisition of meiotic and developmental competence (24). Luvoni et al microinjected 3% Lucifer Yellow solution into canine oocytes collected during different stages of the estrus cycle to evaluate the presence of cumulus-oocyte communications through gap junctions (18). For oocytes collected during late proestrus, 89% (16/18) exhibited open cumulus-oocyte communications through gap junctions, compared to 0% (0/20) for oocytes collected during anestrus (18). When using canine oocytes obtained during anestrus in a culture system, effective media and culture conditions induce open communications between the oocyte and cumulus cells.

OOCYTE NUCLEAR AND CYTOPLASMIC MATURATION

IVM work in the dog has focused mainly on oocyte nuclear maturation. Very little research has examined the cytoplasmic maturation of the canine oocyte and the important role in fertilization that these cytoplasmic components may play. Recent research in other species shows the important role oocytes' cytoplasmic components play to induce oocyte activation during fertilization (5). Species examined for these studies by Ducibella (mouse, bovine, pig and human), all ovulate a mature oocyte, unlike the dog. For most mammals, the newly ovulated oocyte (at MII) is activated in response to fertilization by a sperm, reacting as follows: cortical granule release to establish polyspermy block, reinitiation of cell cycle, pronucleus formation, and protein synthesis (5). Changes going on inside of the oocyte before and upon reaching nuclear maturation affect the oocyte's ability to release and respond to intracellular calcium; Ducibella reported cytoplasmic components including calcium levels and cortical granual arrangement of the oocytes during immature nuclear status (pre-ovulation for the animals used) were altered when compared to these structures in the mature MII (post ovulation for his animal) oocyte (5). As the oocyte approaches MII, previously clustered cortical granules disperse and migrate close to the cell membrane. At fertilization, elevation in cystolic calcium stimulates a PIP2, IP3, DAG second messenger system which will cause the cortical granule exocytation, resulting in a block to prevent polyspermy , and cell cycle resumption including new protein synthesis (5).

The entry of cells into M (mitosis or meiosis) phase of the cells cycle is initiated by protein kinases referred to as maturation-promoting factor (MPF) (16). MPF contains two subunits termed the catalytic and regulatory subunits. The catalytic subunit transfers a phosphate group from ATP to specific serine and threonine residues of specific protein substrates. The regulatory subunit is referred to as cyclin. cell cycle (16). When the cyclin concentration is low, kinase lacks the cyclin subunit causing to be inactive (16). As the cyclin concentration rises, the kinase is activated and the cell enters the M phase of the cell

cycle. Ducibella hypothesized that "higher [MAP] kinase levels of newly ovulated eggs require multiple calcium oscillation [prior to fertilization] to drive their levels below threshold for cell cycle resumption" (5). In contrast, older ovulated oocytes have a decrease in MAP kinase levels and need only a single calcium rise for activation (5). For mouse oocytes which have reached MII, a single or very few calcium spikes are enough to stimulate cortical granule exocytosis; at other points calcium oscillations are seen (5). The amplitude, duration, frequency and number of calcium oscillation the oocyte is exposed to may have a rolein encouraging the oocyte activation response (5).

Ducibella observed that preovulatory oocytes (prior to maturing to MII) could be penetrated by sperm but "failed to undergo normal activation in many species" (5). He attributed "cortical granule migration to subplasmal position during meiotic maturation" as the reason for the inability of preovulatory (immature nuclear components) oocytes to undergo cortical granule release and to establish zona pellucida block (5). Sperm penetration of these immature oocytes resulted in a high rate of polyspermy (5). Mouse and human oocytes at the germinal vesicle stage have a large number of cortical granules in their cortex, "which fail to undergo release after fertilization" (5). "The signaling pathway required of cortical granule exocytosis may become fully competent only after meiotic maturation to MII complete" (5). In vivo, canine fertilization studies by Van der Stricht, as early as 1923, report sperm penetration of oocytes at several stages of maturity including the dictyotene (germinal vesicle) stage (26). Austin and Short and Van der Stricht report that dog and fox spermatozoa may penetrate oocytes at any stage of development post ovulation but the sperm head remains almost unchanged until extrusion of the second polar body (1,26). Fresh ejaculated canine sperm can remain viable up to 268 hours in the estrus bitch's reproductive tract; mating resulting in fertilization can occur from 3 days before until a week after ovulation (19,4,28). Noting that the first polar body is extruded 3 days after ovulation in vivo, while the oocyte is in the oviduct, compared to the longevity of canine sperm once in the estrus female reproductive tract, it is likely that sperm come in contact with immature

canine oocytes in vivo (25,19,28). Mahi and Yanagamichi report “the vitellus of dictyate oocytes to not only be penetrable by spermatozoa , but also capable of decondensing sperm nuclei” (19). Dictyate oocytes capable of penetration by spermatozoa, sperm nuclei decondensation, and formation of male pronuclei would be a useful tool in developing the canine in vitro fertilization model especially considering the difficulties encountered in maturing canine oocytes in vitro. Similar to Mahi and Yanagimachi’s reports, Hewitt and England have published that “the effect of nuclear maturation of oocytes had no effect upon spermatozoal penetration and immature oocytes could be penetrated by spermatozoa in the canine” although is important note that to date, only one pregnancy, which last past 21 days, and no puppies born from in vitro fertilized canine oocytes of any stage of meiotic maturity (10).

Oocyte nuclear maturation and cytoplasmic maturation are considered independent events that occur in vivo during concurrent times. In mouse oocytes, a failure of cytoplasmic maturation can result in MI or MII arrested oocytes that have lower rates of fertilization and development to the blastocyst stage (5). In humans, incomplete cytoplasmic activation has been recognized as the cause of failed fertilization after Intra Cytoplasmic Sperm Injection (ICSI) for Metaphase II oocytes collected after exogenous hormonal stimulation (5). Changes in zona pellucida morphology, and an increased sensitivity of IP3 receptors in the oocyte are two other cytoplasmic changes that occur as the oocyte proceeds from the germinal vesicle to MII during maturation (5). Although reports have stated that immature canine oocytes can be penetrated by sperm, none fertilized in vitro have resulted in term embryos or live births which may indicate that mature cyoplasmic components are necessary to produce a sustainable canine embryo.

REPRODUCTIVE HORMONE SERUM CONCENTRATIONS IN THE BITCH

Concannon closely examined the reproductive hormone cycles of a colony of female beagles over ten years (3). These studies reported average serum levels of hormones important during phases of the bitch estrous cycle. The ovarian cycle is classified by three phases known as the follicular phase, ovulation and the luteal phase (3). The canine follicular phase can persist for one to three weeks and is associated with release of the anterior pituitary hormone FSH which stimulates follicle growth, resulting in increasing levels of estradiol produced by graulosa cells of the growing follicle. The serum concentration of estradiol increases during the follicular phase of the cycle from basal levels of 2 to 10 pg/ml to the highest serum concentrations of 50 to 120 pg/ml (3). The known affects of estrogen include an increase in length and folding within the uterine horn, stimulation of endometrial hyperplasia, endometrial secretion of proteins, thickening of the vaginal wall, and enlargement of the cervix (3). The bitch's progression from proestrus into estrus is usually noted by receptivity to the male, or standing for copulation. Plasma estradiol 17-ß levels rise during proestrus and decline during sexual receptivity (3). During the proestrus follicular phase, the bitch is usually not receptive to mounting behavior by male dogs.

As serum concentrations of LH increase from basal plasma concentrations ranging from 21 to 156 ng/ml , estradiol levels begin to decrease, LH surges (mean plasma concentration of 402 ng/ml) during late proestrus or early estrus, causing ovulation and commences the start of the luteal phase of the cycle (3), Frequently the day of the LH peak can be correlated to the same day or one day after the first time progesterone levels rise above 1.0 ng/ml, compared to 0.4 to 0.6 ng/ml during proestrus, and between 0.8 and 1.2 ng/ml during peak estrogen levels. While progesterone levels increase steadily during the LH surge, serum estrogen levels are simultaneously falling. After the LH peak, basal levels of LH are restored; ovulation in the bitch occurs two days after the LH surge (3). Maximum serum concentrations of FSH are 297$\pm$ 26 ng/ml occur the day after serum maximum of

LH. Four to eight days post LH peak, canine serum FSH concentrations decline to below 200 ng/ml (22).

The luteal phase is noted by high levels of progesterone to maintain pregnancy if fertilization does or does not to occur. Hewitt and England 1997 reported serum progesterone levels of 4 to 8 ng/ml two days after the LH surge, on the day of ovulation (8). Post ovulation, serum progesterone levels in the bitch continue to rise reaching a peak of 15-30 ng/ml, occurring 15 to 25 days after the LH peak (3). During the peak of serum progesterone levels, plasma estradiol concentrations have been reported to be in the range of 0.8 to 1.2 ng/ml (3,8).

BASIC IN VITRO MATURATION MEDIA

In recent years much attention has been focused on developing a defined media in which to mature canine oocytes in vitro. Assisted reproductive techniques (ART) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), have been used successfully in clinical settings for a variety of species. Typically these require the use of an oocyte matured to the metaphase II stage of nuclear development. Local veterinary clinics performing routine spay surgeries provide a ready supply of canine ovaries from bitches of all ages, breeds and in all stages of the estrous cycle. Immature oocytes to be used for experiments were obtained by slicing ovaries removed from bitches during routine ovariohysterectomy (1). Developing a defined media in which to in vitro culture these oocytes to mature them to the MII stage before being introduced to sperm should logically increase success rates of obtaining multicellular embryos for embryo transfer. Advanced nuclear maturation at the time of sperm/oocyte interaction may be necessary to produce an in vitro fertilized canine oocyte capable of development to the morula or blastocyst stage to be used for embryo transfer to a primed canine uterus. The only in vitro fertilized canine pregnancy, reported by England et al 2001, utilized oocytes obtained from a bitch in estrus (7).

Many in vitro maturation (IVM) medias developed for use in other species contain 10% or 20% heat-inactivated fetal bovine serum (FBS). Generally, researchers strive to use completely defined medias to assure that conditions can be repeated exactly, this discourages the use of a serum supplemented media . Supplementing IVM medias with serum collected from a female of the specific species while in estrus often enhances in vitro oocyte nuclear maturation. In the horse, a tissue culture media (TCM) 199 supplemented with 15% serum collected from a mare on her day of ovulation, has been shown to mature oocytes that were 80% GV at time 0, to 47% MII after 15 hours of in vitro culture (27). Personal communications with Fulton indicate obtaining 36% of canine oocytes reaching MII when in vitro matured for 72 hours in an TCM-199 plus 20% estrous bitch serum media (personal communications, Fulton, 1998). The goal of developing a completely defined media discourages the use of culture media supplemented with estrus serum due to variability between females even within the same species.

For serum supplementation experiments conducted by Hewitt and England, their basic culture media contained the following: Modified Hepes buffered TCM-199 (containing 100 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mg/ml sodium bicarbonate) supplemented with either 5, 10 or 20% FBS, or 0.3 or 0.4% bovine serum albumin (BSA) (fraction V, Sigma) (11). Control oocytes were treated without FBS or BSA. Oocytes were obtained from ovaries in various stages of the estrous cycle (routine spays), and were cultured in the various test medias for 0, 48 and 96 hours at 39°C, 5% CO_2 (14).

Hewitt reported media supplemented with BSA as preferable for in vitro culture of canine oocytes in comparison to FBS supplements which resulted in significantly large numbers of canine oocytes with unidentifiable nuclear matter (14). In this study, the highest percentage of oocytes maturing to the MI/AI/MII stage was 36% (12 out of 33); these oocytes were treated with the basic culture media supplemented with 0.3% BSA for 48 hours (14).

IVM MEDIAS SUPPLEMENTED WITH HORMONES

Success rates associated with in vitro maturation of oocytes and the development of IVF embryos for many species have been greatly improved by the addition of various hormones to IVM medias. Zuelke and Brackett examined the effect of LH supplemented media for maturing bovine oocytes in vitro. Immature bovine oocytes were treated with a modified TCM-199 supplemented with 10 µg/ml, 50 µg/ml or 100 µg/ml of LH (29). Oocytes were incubated in one of the three IVM test media for 24 hours, followed by fertilization by co-incubation with sperm for 24 hours. After sperm co-incubation, the oocytes were cultured on cumulus cell monolayers; embryos were assessed for development eight days post insemination. Control oocytes were treated in the same manner using an IVM media containing no LH supplement. In vitro maturation media supplemented with LH improved bovine embryonic development, "embryonic viability (reaching blastocyst after 8 days post insemination) improved significantly after IVM with either 50 µg LH/ml (27%) or 100 µg LH/ml (28.3%) versus TCM-199 alone (9.9%) or 10 µg LH/ml (10.8%)" (29).

Experiments reported by Hewitt and England compared maturation rates of canine oocytes obtained from bitches at various stages of the estrous cycle cultured Hewitt and England basic culture media supplemented with 1µg/ml LH, 1 µg/ml FSH, or 1 µg/ml LH along with 1 µg/ml FSH (13). Control oocytes were treated with Hewitt and England's basic culture media containing 0.3% BSA with no gonadotropin supplements (13). Oocytes were cultured in the various test media for 48 and 96 hours and showed no significant difference in percentage of canine oocytes to mature to GVBD or MI/AI/MII between any of the treatment groups (13). Two treatment groups from the 48 hour incubation trial contained oocytes matured to MI/AI/MII; these were 2 out of 25 (8%) for the LH group and 1 out of 34 (3%) for the LH in combination with FSH group (13). For the 96 hour culture trials, 3 oocytes out of 25 (12%) from the FSH supplemented group matured to MI/AI/MI (13). For this experiment, the effect of only one concentration of each gonadotropin was

examined while in vivo, oocytes are exposed to multiple gonadotropins in conjunction with steroid hormones at varying concentrations (13).

Hewitt and England examined the role of steroid hormones on canine oocyte IVM by culturing oocytes from late luteal or anestrus bitches in Hewitt and England basic culture media containing 0.3% BSA supplemented with 1µg/ml estradiol, 1 µg/ml progesterone, or 1 µg/ml estradiol along with 1 µg/ml progesterone. Oocytes were cultured at 39°C, 5% CO_2 for 48 and 96 hours. Control oocytes were treated in the same manner but with no steroid hormone supplements. The most successful group- 20% of the oocytes to reach MI/AI/MII- was the group cultured for 48 hours in IVM supplemented with a combination of estradiol and progesterone. These findings were not statistically significant; no significant difference was recorded for oocytes reaching MI/AI/MII between varying IVM time lengths and hormones used as a supplement. The published results for this experiments gives only percentages, not numerical data. It should be noted that an average of only 28 oocytes were used per trial and only one concentration of the hormones was evaluated.

Durrant examined the effect of an IVM containing both a gonadotropin and steroid hormone on maturation of canine oocytes obtained from advanced preantral and early antral follicles (6). The IVM media consists of the following: Dulbecco's Modified Eagle's medium (DMEM) nutrient mixture F-12 Ham culture medium supplemented with 20% FBS, 2 mM L-glutamine, 1% antibiotic-antimycotic, 1 µg/ml FSH, 10 IU/ml hCG, 1 µg/ml estradiol 17-ß. Oocytes were cultured in the IVM media for 24, 48 or 72 hours at 37°C, 5% $C0_2$ (6). As a control, a group of oocytes was stained for nuclear assessment at zero time with no additional incubation.

Durrant reported that the largest group of oocytes to reach MI/AI/MII was 47 oocytes out of 407 (11.5%) for the 48 hour incubation. The 72 hour incubation resulted in 40 out of 404 (9.9%) of the treated oocytes to mature to MI/AI/MII. While large numbers of oocytes were used for this experiment but it would have been beneficial to have included a control

group of oocytes incorporating the same IVM culture periods as treated oocytes but in a non hormone supplemented IVM media.

Further research is needed to assess the effect of various gonadotropin and steroid hormones on IVM of canine oocytes. The development of a reproducible canine IVM and IVF systems will likely involve the use of multiple hormone supplements at various concentrations to more closely mimic the in vivo environment during canine ovulation and fertilization.

EXPERIMENTS RESULTING IN VITRO FERTILIZED CANINE EMBRYOS

Yamada et al reported obtaining the following embryos after IVF in his 1992 experiment: 1 eight cell, 2 seven cell, 2 six cell, 1 five cell, 4 four cell, 1 three cell, and 4 two cell embryos. Oocytes and ovaries from fifty-four bitches all treated with the following superovulation regimen were used: 1) estrone (100-400 μg/day) injected daily until vaginal discharge was detected; 2) three days after onset of vaginal discharge, eCG (400 IU) and hCG (1000 IU) were injected s.c.; 3) two injections of estradiol (10 μg/day), given i.m., 3 days and one injection at 4 days after the eCG and hCG injections; 4) estrus as determined by vaginal cytology and percent of cornification, on the first day of estrus hCG, 1000 IU was injected iv; 5) ovaries were collected 72 to 78 hours after iv hCG. Ovulation occurred in the bitches 72 hours after intravenous hCG injection. After obtaining oocytes from these superovulated dogs, oocytes were placed in a culture media containing 10% FBS. At the end of 72 hours IVM, 31.9% of oocytes had matured to MII.

Sperm solutions were prepared by centrifuging and capacitated with BSA prior to being added to the culture media containing the 72 hour IVM oocytes (28). After co-incubating the oocytes with the capacitated sperm for 18 to 20 hours, COCs were returned to culture for an additional 48 hours. At this time, 14% of the fertilized COCs had developed into embryos at the two to three cell stage. Seventy-two hours post insemination 4.8% (3

out of 62) of fertilized COCs had resulted in five to eight cell embryos, 29% were two to four cell. Ninety-six hours post insemination 13.3% of fertilized COC had developed beyond the 4 cell embryo stage. No development of blastocysts or morulas occurred.

Hewitt and England have reported development of one two cell embryo obtained during a study of canine oocyte penetration (10). Plasma progesterone levels of experimental dogs were monitored; the first day plasma progesterone levels exceeded 3 ng/ml, ovariohysterectomy was performed (10). Ovaries were sliced to release oocytes into modified M199 and graded on a scale of 1 to 3 with grade 1 being darkly pigmented and completely surrounded by one or more layers of cumulus cells, grade 2 lightly pigmented with incomplete layers of cumulus cells, and grade 3 being degenerate and exhibiting pale color and frequently misshaped and without any cumulus cell attached. The two cell embryo produced and published during this study was a result of IVM in Hewitt culture media (modified M199 with 0.3% BSA) at 39°C for 72 hours, co-incubation for 12 hours with sperm that had been treated with CCM, followed by a final 48 hours of culture for the fertilized oocyte (10). Note that the majority of multi cell embryos containing more than 2 cells reported by Yamada, were seen between 72 and 96 hours post insemination (28). Upon further examination, Hewitt found that 75% of oocytes remaining at GV stage when introduced to sperm were penetrated by the sperm (10). Of oocytes that had resumed or completed nuclear maturation (GVBD, MI, AI, MII), 88% were penetrated by sperm (10). No significant difference between these two numbers leads to Hewitt's conclusions that "the stage of nuclear maturation of the oocyte doesn't affect spermatozoa ability to penetrate the oocyte" (10).

While Hewitt and Yamada both comment that ability of spermatozoa to penetrate canine oocytes has no dependence on nuclear maturation of the oocyte, while these oocytes may not have achieved nuclear maturation during the preinsemenation 48 to 72 hour culture period, other chemical and cytoplasmic changes may have taken place in the oocyte which

may have an affect on its ability to be fertilized and develop into a multicellular embryo. Also it is important to note that oocytes obtained by Yamada and Hewitt were both "primed" in vivo during estrus with removal of ovaries by surgery just hours before ovulation was to naturally occur; oocytes obtained from bitches in various stages of the estrus cycle may not result in effective embryo production.

Using oocytes collected from bitches in various stages of the estrous cycle, Otoi et al reported in 2000 the development of blastocyst. In vitro maturation was performed with COCs being cultured on a feeder layer of bovine cumulus cells in a culture medium in which in vitro fertilized bovine embryos had been cultured for 13 to 14 days (23). Oocytes were cultured for 72 hours at 37.5^{o}C, 5% CO_2 on the bovine cumulus cells. As a control, a group of COCs were cultured without the bovine cumulus cells. Ejaculate was centrifuged followed by spermatozoa being washed and diluted to a 1×10^6 concentration in Brackett-Oliphant medium containing bovine serum albumin (3 mg/ml), heparin (10 µg/ml) and caffeine (2.5 mM) (23). After the 72 hours of IVM culture, the COCs were cultured with the sperm suspension for an additional 6 hours. Following co-incubation with sperm, COCs were moved to a feeder layer of bovine cumulus cells for 10 days of culture. At this time, one blastocyst was reported (23).

While live births have resulted from IVF oocytes in the cow, human, mouse, pig, goat , sheep and cat, to date, none have been reported in the dog. The most advanced report of an IVF canine embryo is England's recently published 22 day pregnancy (7). The oocytes resulting in the pregnancy were obtained from research bitches originally in anestrous and treated daily with cabergoline (a prolactin inhibitor that induces estrus) for 12 to 15 days, until the onset of vulva bleeding. At this point, daily vaginal cytology and plasma progesterone levels were recorded. Once plasma progesterone concentrations exceeded 5.0 ng/ml, ovariohysterectomy was performed to obtain oocytes.

After being removed from the ovaries of the research bitches, 169 grade I cumulus oocytes complexes (COCs) (darkly pigmented and completely surrounded by one or more

layers of cumulus cells) were cultured in TCM 199 containing 0.3% BSA for 24 to 78 hours (7). Ejaculate was collected and treated with canine capacitation media and added to the cultured COCs for 12 hours (7, 20). Post sperm incubation, oocytes were cultured for another 48 hours. At this point, two out of 90 oocytes were at the two-cell embryo stage. All 90 embryos were surgically transferred to the uterine lumen of a surrogate bitch whose plasma progesterone was above 5.0 ng/ml. Researchers used laparotomy along with a catheter and sterile glass pipette to place the embryos into the surrogate bitch's uterine lumen. In vivo, embryos descend from the oviduct into the uterine horn at the morula stage of embryo development. The surrogate bitch was administered ampicillin and 30 mg of progesterone in oil at the time of embryo transfer. Ultrasound was performed 20 days post embryo transfer to confirm pregnancy. At this time, one conceptus was in the proximal region of the uterine body and two conceptuses in the left uterine horn. Researchers noted that all three conceptuses were considered to be undersized for their anticipated age. The next ultrasound, at 22 days post embryo transfer, all conceptus were absent. The surrogate bitch's serum levels for progesterone , relaxin and fibrinogen were noted as normal for early pregnancy.

EVALUATION OF OOCYTE NUCLEAR MATURATION

Many investigators use an aceto orcein staining dye for nuclear assessment of canine oocytes. To visualize oocytes, cumulus cells must be removed which can be achieved by 20 minutes in a hyaluronidase solution followed by 10 minutes of high vortexing (unpublished Dookwah, Dew and Baracaldo 2000). Oocytes are then mounted on a slide and a coverslip is placed by parafin-vassaline support columns to allow a compression system. The slide is treated with Acetic acid(3): methanol (6): Chloroform (2) for 2-3 minutes (14). A 48 hour treatment of Acetic Acid (1):Methanol (3) is used to aid in clearing the canine oocyte's dense lipid, after which staining is performed with 1% aceto orcein in 45% Acetic Acid,

slides are treated with a 20% glycerol, 20% Acetic Acid, 60% distilled water solution (14).

Fluorescent dyes such as Hoescht 33342 have proven successful for use in nuclear assessment of the canine oocyte. This dye allows for visualization of the nuclear material but does not permit distinction of individual chromosomes like the aceto orcein stain. The benefits of Hoescht 33342 are that it is a much faster staining process (2O minutes) and will not cause cell death as is associated with the aceto orcein. Using a UV lamp on and inverted microscope allows nuclear viewing of oocytes with a Hoescht 33342 stain.

DEGENERATE OOCYTES

An alarmingly large percentage (65%) of cultured oocytes which were harvested from canine ovaries removed from the bitch during random phases of the estrous cycle (obtained from local veterinary clinics during routine spays) have been noted to be degenerate (19). Surprisingly, this number decreased only minimally, to 62.6%, when a superovulatory regime of estrone, equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), and estradiol was given just prior to removing the ovaries and harvesting oocytes (28). Yamada states that “it is unclear whether these arrested oocytes were derived from follicles not destined to ovulate or were due to incomplete culture conditions” (28). Dramatically decreasing the number of degenerate oocytes obtained, or rather increasing the number of viable oocytes harvested will be crucial when using these techniques in future clinical cases.

Hewitt and England reported similar experiences after culturing oocytes from ovaries removed from a dog just hours prior to ovulation versus various times in the estrous cycle (9). Hewitt and England reported examining oocytes obtained from the ovaries of 68 bitches of various breeds, between one and seven years of age, and at various stages of the estrus cycle (9). Fifteen percent (68 out of 462) of grade I COCs examined by Hewitt and England in the 1998 experiment were labeled as non-identifiable nuclear material (9). Hewitt and England propose that the non-identifiable nuclear material oocytes may “reflect a transitory

stage in the nuclear maturation cycle or alternatively , it may be that these oocytes were obtained from atretic follicles" (9). They reported no difference in the number of oocytes undergoing nuclear maturation while in in vitro culture from ovaries obtained just prior to natural ovulation compared to ovaries obtained at various stages of the estrous cycle (9). These findings are difficult to compare to Yamada because of Yamada's use of a superovulation regiment on all bitches in the study. No use by Yamada of data collected from non-superovulated bitches, as a control, makes it difficult to effectively evaluate superovulation.

CLINICAL IMPLICATIONS AND FUTURE USES

In 1982, the first live birth of the first cow resulting from IVF was reported (2). Since then, IVF efficiency in the cow has increased dramatically due to the large number of advances in the field. The perfection of an ART system which would enable researchers to produce live birth puppies has definite clinical applications. ICSI is used successfully in the human to produce pregnancies in cases of male orchitis, trauma, testicular degeneration and senility. For cases of very few sperm cells and sperm cells of poor quality (such as oligospermia), with the use of frozen sperm from a deceased stud dog, or in emergency cases of post mortal sperm collection, ICSI is an extremely important tool to produce maximum embryo production efficiency.

In dealing with infertility of the bitch, especially when associated with pyometra, IVF along with embryo transfer involving the use of a surrogate uterus provides potential for future progeny after removal of a bitch's uterus and ovaries. Pyometra results from a uterine infection which can threaten the life of the bitch, all of the uterus must be removed to prevent stump pyometra. Ovaries can possibly be removed from a bitch facing pyometra and cryopreserved for future IVF and embryo transfer. Until further improvement in oocyte cryopreservation, researchers currently recommend that ovaries be left intact to

harvest oocytes at a later date for assisted reproduction (personal communication, R. A. Fayrer-Hosken) with entire uterine removal.

ART has already proved successful in producing live young from endangered felids; this same technology may assist reproduction of endangered canids and foxes in the future. These reproductive technologies can help to preserve genetic material, or bring new genes into an area. Embryo transfer would allow a completely unrelated individual to be born into new community, avoiding pack acceptance problems often seen when attempting to introduce a foreign infant or adult, while providing completely new genes into the pack.

The laboratory of Ian Wilmut produced the first live mammalian offspring, the sheep "Dolly", following nuclear transfer from an established cell line in 1996. Cloning, which requires the use of IVM and embryo transfer techniques, could be a helpful tool in genetic research by assisting in the creation a greater numbers of animals affected with certain genetic diseases, creating a population large enough to be effectively studied.

Current ART techniques such as IVF, ICSI, and nuclear transfer require the use of an oocyte at MII. Development of a reliable in vitro system for the maturation of canine oocytes to MII would make the latest in reproductive technology clinically applicable for the dog. Our overall goal is to be able to help caring and concerned dog owners and provide them with alternatives when dealing with the emotional distress of facing infertility in their companion animals.

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CHAPTER II

THE EFFECTS OF PROGESTERONE, ESTRADIOL, rFSH, eCG, L-CYSTEINE AND ARACHIDONIC ACID ON CANINE OOCYTE IVM

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ABSTRACT

Assisted reproduction technologies (ART) has been successfully applied to species including man. These techniques are still to be refined for clinical application in canines. The need for ART include the conservation of genetic material of endangered animals as well as therapies for treatment of canine infertility. Success of canine in vitro embryo production has been extremely limited; there has been one report of a canine pregnancy from IVF, however, this has not resulted in term pregnancies or births of live puppies. Unlike other mammals, the dog ovulates a primary oocyte, which requires further maturation to metaphase II (MII) within the oviduct. Medias supplemented with steroid hormones, gonadotropins, antioxidants and fatty acids have enhanced oocyte IVM rates for a variety of species. Our objective was to examine more fully the role of estradiol, progesterone, FSH, eCG, LH, L-cysteine and arachidonic acid on in vitro maturation of canine oocytes and to develop a reproducible in vitro maturation technique for canine oocytes. Oocytes harvested from bitches undergoing routine ovariohysterectomy were cultured in a basic culture medium with or without supplementation. For experiments examining the effects of estradiol, progesterone, FSH and eCG, oocytes were cultured with one of three concentrations of the hormone or no hormone supplement over 24, 48, 72 or 96 hours. To examine the effect of rFSH, oocytes cultured in media supplemented with 0.1 IU/ml, 1.0 IU/ml, or 10.0 IU/ml of rFSH or no hormone supplement over 24, 48, 72 or 96 hours were examined. For experiment examining the effects of LH, oocytes were cultured with one of two concentrations of the gonadotropin or the control treatment for 96 hours. For experiments examining the antioxidant L-cysteine or fatty acid arachidonic acid, two concentrations were compared to the control treatment over 72 hours of culture. After culture, oocytes were stained with Hoechst 33342 and classified into one of the following five stages of nuclear maturation: germinal vesicle (GV, tight compact nucleus), germinal vesicle break down (GVBD, nucleus less compact), metaphase I/anaphase I (MI/AI, presence of a

metaphase plate/ chromatids separating), metaphase II (MII, extrusion of a polar body) or unidentifiable (U, unclassifiable nuclear material).

A total of 8,536 canine ooyctes were cultured and examined during this study. The maturation rates of oocytes cultured in any of the rFSH treatments was statistical significant ($p<0.05$) compared to the control treatment although the effect of concentration of rFSH was not shown to be significant. The oocytes cultured for 96 hours in a rFSH supplemented media were significantly more likely to mature to MII than the control group. Medias supplemented with estradiol, progesterone, FSH, eCG, LH, L-cysteine and arachidonic acid did not significantly effect mature rates for canine oocytes when compared to the control treatment.

INTRODUCTION

Assisted reproductive technologies (ART) have been well developed for a variety of species including man. ART techniques, using a mature MII oocyte, such as in vitro fertilization (IVF) and intra cytoplasmic sperm injection (ICSI) have resulted in healthy live offspring for many species allowing this technology to be used regularly in clinical settings. The success of canine in vitro embryo production has been extremely limited, there has been one report of a canine pregnancy from in vitro fertilization but no pregnancies to term or births have been reported (8). Interest in improving assisted reproduction technologies (ART) for the dog has increased particularly in relation to potential application to salvage endangered species and cloning (9,10,23).

The use of ART and cloning techniques developed for other species utilize a mature MII oocyte. Typically, all preovulatory oocytes obtained for culture studies are meiotically immature. In vitro maturation protocols for many species such as the bovine have been very successful resulting in 80-90% of oocytes reaching MII within 72 hours (22). In contrast,

canine oocytes achieving full nuclear meiotic maturation in in vitro culture systems range from 0 to 39% (2,7,15,16,17,27,31,43).

Development of an in vitro system to mature and fertilize canine oocytes has many obstacles which have been resolved for other species (9). These differences may be related to the fact that most mammals ovulate metaphase II oocytes ready for fertilization while the dog ovulates an immature oocyte at the germinal vesicle (GV) stage (18,27,43,44). The canine oocyte resumes meiotic division within the oviduct over 2-5 days post ovulation (18,23,41). This physiologic difference between the dog and other mammals may be an important consideration in the development of an in vitro maturation and fertilization system.

The ovarian cycle is calssified into three phases known as the follicular, ovulation and luteal phases. The follicular phase of the ovarian cycle is associated with an increasing serum concentration of the steroid hormone estradiol, which begins to decline as concentrations of luteinizing hormone increase. The release of the gonadotropin LH from the anterior pituitary, is associated with changes that take place within the follicle including resumption of meiosis for most mammallian oocytes (12). Progression from the follicular into the luteal phase of the bitch’s cycle occurs during the LH surge which takes place during late proestrus or early estrus (5). Serum concentrations of the steroid hormone progesterone begin to rise as LH reaches its peak; from 0.8-1.2 ng/ml during peak estrogen levels to 2-3 ng/ml at the time of the luteinizing hormone (LH) peak (5). Ovulation of the canine oocyte occurs 1.5 to 2 days after the LH peak, when serum progesterone levels are as high as 3-8 ng/ml (5). In vivo, the oocyte passes from the ovary into the oviduct after ovulation and once in the oviduct, the oocyte will mature to MII over the next 2 to 5 days while serum concentrations of progesterone increase and reach a peak of 15-80 ng/ml between 15 and 25 days after the LH peak (5,8,23,41).

The luteal phase of the cycle, which occurs after ovulation, is noted by high levels of progesterone to maintain pregnancy if fertilization were to occur (5, 33). In contrast to

most species, luteinization of the bitch's ovarian follicle begins prior to ovulation. It has been hypothesized that this is related to the long interval between the LH surge and ovulation (32). LH, along with prolactin, plays a role in maintaining the corpus luteum and progesterone production throughout pregnancy (32). LH receptors remain on the surface of the canine corpora lutea in constant concentration throughout the estrous cycle (32).

Canine oocytes have been cultured in medias containing 1.0 μg/ml of estradiol, 1.0 μg/ml progesterone, both steroid hormones in combination or no supplement (13). An average of 33 oocytes per treatment group were cultured for 48 or 96 hours. There was no statistical difference for oocytes maturing to MI/AI/MII between oocytes treated with estratiol, progesterone, both steroid hormones or no supplement (13). Bolamba reported canine oocyte IVM using a basic media supplemented with gonadotropins and estradiol over 24, 48 and 72 hours of culture (2). For oocytes cultured for 24 hours, 20 out of 374 (5.3%), for the 48 hours culture group, 47 oocytes out of 407 (11.5%), and for the 72 hours culture group, 40 out of 404 (9.9%) matured to MI/AI/MII which was significantly more MI/AI/MII oocytes than oocytes that were not cultured (2).

In 1990, Zuelke and Brackett examined the effect of LH supplemented medias on the nuclear maturation of bovine oocytes in vitro. In vitro maturation media supplemented with LH aided bovine embryonic development: "embryonic viability (reaching blastocyst after 8 days post insemination) improved significantly after IVM with either 50 μg LH/ml (27%) or 100 μg LH/ml (28.3%) versus TCM-199 alone (9.9%) or 10 μg LH/ml (10.8%)" (48). A similar finding was reported by Younis et al in 1989. The addition of LH to oocyte maturation media containing estradiol greatly enhanced development of four and eight cell embryos in vitro produced from matured bovine embryos (46).

Hewitt and England enhanced maturation rates of canine oocytes cultured in media supplemented with LH alone and to oocytes cultured in media containing LH along with FSH. Their results showed no significant difference in percentage of canine oocytes maturing to GVBD or MI/AI/MII in any of the treatment groups (16). Only one concentration of

each gonadotropin was examined. In vivo, oocytes are exposed to multiple gonadotropins in conjunction with steroid hormones at varying concentrations (16). Studies examining the effect of an LH supplemented media on in vitro maturation of domestic cat oocytes reported a significantly greater percentage of oocytes cultured with gonadotropins matured to MII when compared to a control (38). A significantly greater percentage (31%) of oocytes cultured in media containing FSH and LH matured to MII compared to 20% of the oocytes treated with media containing no hormone supplement (38).

LH has been shown to disrupt gap junctions existing between cumulus cells and oocytes in the fox (19). Two to three days after the LH peak, when metaphase I was reached, junctions which were present between cumulus cell projections and the oocyte before, had become disrupted. Krogenaes et al reported that viable and differentiated cumulus cells were needed for the resumption of meiosis for cultured fox oocytes (21).

Serum concentration of the gonadotropin follicle stimulating hormone (FSH) for bitches in anestrus remains relatively constant with a range of 240- 294 ng/ml (33). During proestrus, serum concentration of FSH declines, but rises one or two days after the LH peak to levels similar to those seen during anestrus. For the remainder of estrus, the serum concentration of FSH declines; with the mean value during late estrus being less than 200 ng/ml (33). In other species such as the goat, immature oocytes have been successfully matured in media containing FSH, fertilized in vitro, and have resulted in term embryos and live births (8,47). Hewitt and England cultured canine oocytes in media supplemented with 1 μg/ml LH, 1 μg/ml FSH, or 1 μg/ml LH and 1 μg/ml FSH over 48 and 96 hours (16). Oocytes categorized as MI/AI/MII resulted from the 48 hour LH treatment (2/25), the 96 hour FSH treatment (3/25) and the 48 hour LH and FSH treatment (1/34) although there was no statistically significant difference between the control and treatment groups (16).

Blue Fox oocytes cultured in the presence of FSH showed an increased percentage of maturation to MII when compared to oocytes cultured without FSH (21). Significantly

more oocytes cultured for 48 and 72 hours in media supplemented with 2.5 µg/ml FSH matured to MII (11%, and 21% respectively) than oocytes cultured in control media for 48 hours (2%) and 72 hours (11%). Culture lengths of 24 and 96 hours in this study did not produce significantly more MII oocytes than control groups.

During maturation and fertilizaton, the mammalian oocyte is exposed to various gonadotropins within the ovary and the oviduct. In the pregnant mare, eCG is produced by the endometrial cups which start to develop on day 36 of pregnancy. This gonadotropin is comprised of an a and ß subunit protein. In vitro, eCG has been attributed to stimulating progesterone production through the primary and secondary corpora lutea (40). eCG has also been noted as having both LH and FSH activities in gonadal tissue of the rat, mouse, pig and cow (40). Equine chorionic gonadotropin (eCG) has been used successfully in a number of species to induce estrus (42). Anestrus bitches treated with an intramuscular injection of eCG (20 IU/kg) daily for five consecutive days (referred to as days one through five) followed by one 500 IU intramuscular injection of human chorionic gonadotropin (hCG) came into heat by days nine to fifteen (42). Oocytes obtained from cows prestimulated with eCG 24 hours before slaughter, have been successfully matured and fertilized in vitro, resulting in live births (34).

In vitro production of embryos developing up to the eight cell stage has been reported in conjunction with the use of in vivo superovulation regimen that included estrone injections prior to harvesting oocytes (43). Hewitt and England reported a two cell embryo produced by IVF of oocytes retrieved from spay surgery on a bitch whose plasma progesterone levels had exceeded 3 ng/ml (14). In vitro fertilized oocytes resulting in the pregnancy recently reported by England et al, were retrieved from bitches with plasma progesterone concentrations exceeding 5.0 ng/ml within a 36 hr period (8).

Another concern when developing IVM and IVF systems involves free radical reactions. During metabolism, free radicals and reactive oxygen intermediates such as perox-

ides are formed. Free radical reactions are known to cause inactivation of enzymes and alteration of membranes and DNA (47). The tripeptide glutathione (gamma-glutamylcysteinylglycine; GSH) is the “major nonprotein sulphydryl compound in mammalian cells”; it plays a role in protecting cells against the effects of free radicals and reactive oxygen intermediates (6,29). The many reported actions of GSH include, reduction of disulfide bonds and effects on DNA synthesis, protein synthesis, and amino acid transport (6). Another activity of GSH involves breaking down disulfide bonds, thought to play a role in decondensation of sperm heads just after fertilization (36). For the mouse and hamster, GSH synthesis must occur during oocyte maturation for later sperm chromatin decondensation and male pronucleus formation within oocytes after sperm penetration (3,35,36). Mature metaphase II hamster oocytes exhibit significantly higher levels of GSH (8mM) than germinal vesicle (4mM) or pronuclear (6mM) oocytes (36). Researchers in the field have hypothesized that the “addition of substances that stimulate endogenous systems of cell defense for reducing free radical levels, could be advisable for the establishment of a suitable culture system”; and potentially aid with in vitro maturation of oocytes (25).

Production of GSH occurring during oocyte IVM is reported for many different species including mouse (3), hamster (36), pig (45), and cow (6). Investigators examining the role of GSH on IVM and IVF of porcine oocytes compared oocytes cultured with buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, to a control group of cultured oocytes (45). Oocytes matured in vitro in a BSO supplemented media exhibited substantially lower levels of GSH production compared to control oocytes indicating that GSH production occurs throughout pig oocyte maturation in vitro (45). There was no difference in the rate of cumulus mass expansion before insemination or rates of nuclear maturation and sperm penetration after in vitro insemination when BSO cultured oocytes were compared to controls (45).

Reports from Yoshida’s 1993 experiments with pig oocytes showed that the presence of decondensed sperm heads and male pronucleus formation was significantly higher for

oocytes in the control group compared with those treated with BSO throughout culture or within 12 hours of the start of culture (45). This indicates that oocytes in vitro matured early in the presence of BSO, causing lower levels of GSH synthesis compared to controls, lack sufficient agents to reduce sperm heads later during fertilization (45). These investigators concluded that GSH synthesis occurs throughout in vitro maturation of pig oocytes but is especially important during initial and mid phases of in vitro oocyte maturation to act, following sperm penetration, as a cytoplasmic factor playing a role in regulation of sperm nuclear decondensation and male pronucleus formation (45).

Similar findings have been reported for mouse oocytes. Mouse oocytes cultured in a BSO supplemented media contained 10% less intracellular GSH than control oocytes (3). Mouse oocytes cultured in the BSO and fertilized in vitro resulted in abnormal one cell embryos noted by the female pronucleus coexisting with an unchanged sperm nucleus. This indicates that adequate intracellular GSH levels are necessary for “initiating transformation of the fertilizing sperm nucleus” (3).

GSH synthesis has been shown to require only a media containing cysteine (29). Pope et al reported that the development of cat oocytes matured and fertilized in vitro is enhanced by maturation media containing 0.1 mg/ml of cysteine along with culture in a reduced O_2 atmosphere (37). Luvoni and Colombo 1995 also reported maturation rates were significantly increased for feline oocytes cultured in a maturation media containing 0.1mM of L-cysteine (24). Feline oocytes cultured in the presence of L-cysteine resulted in intracellular GSH levels significantly higher than oocytes cultured without L-cysteine (26). De Matos et al. in 1995 reported blastocyst development was significantly higher for bovine oocytes matured in media containing 100 μM of cysteamine compared to oocytes cultured in media containing no cysteamine (6). Cysteamine acts by reducing cystine to cysteine and promoting the uptake of cysteine which enhances production of GSH (6).

Recent reports indicate that embryos matured and fertilized in vitro have lower lipid contents than those fertilized in vivo, “bovine in vivo produced embryos contain much

more triglycerides and less lipids from other classes than in vitro embryos" (1). Radio labeled [3H] oleic acid was used in these studies. Investigators examining other species have reported that a large amount of the total lipid present in oocytes is in the form of tryglycerides; "the pig oocyte contains 75 ng triglycerides of a total lipid content of 156 ng, which also includes phospholipids, cholsterolesters and free fatty acids" (28).

Studies using microinjection of Xenopus oocytes with three phospholipases which act on phospholipids, showed an inductive effect on oocyte maturation. This effect is the result of an increase in production of second messengers including lysohospholipids, arachidonic acid, diacylglycerol and phoshatidic acid (4). Microinjection of "phosphatidylcholine-hydrolyzing phospholipase C" in Xenopus laevis oocytes has also been shown to induce maturation (11). One related study that used a culture system instead of microinjection is reported by Sorbera et al (39). This group stated that free arachidonic acid placed in the maturation media during culture "induced maturation in a dose and time dependant manner" for european Sea Bass (*dicentrchus laabrax*) oocytes (39). The optimal dosage of arachidonic acid to oocytes in culture was shown to be 100 μM (39).

We are interested in developing an in vitro model for which oocytes at various ages of the estrus cycle, from bitches of various age and breed, can be reliably matured to MII. An effective culture system should provide an environment resembling that of in vivo conditions. Our objective was to examine more fully the efficiency of an in vitro maturation (IVM) media supplemented with progesterone, estradiol, rFSH, eCG, LH, L-cysteine or arachidonic acid on in vitro maturation of canine oocytes over varying culture periods. The effect of supplemented media on maturation of canine oocytes was compared to that of non-supplemented IVM media cultured over the same lengths of time.

MATERIALS AND METHODS

Oocyte Collection

Canine reproductive tracts were collected from bitches of various age and breed via routine ovariohysterectomies performed at local veterinary clinics. Tracts were placed in a Transport Media (DMEM, 1% Penicillin Streptomycin, 0.1% Fungizone) (Sigma, St. Louis, MO) maintained at 38.8^{o}C. Tracts were brought to the laboratory within 4 hours of surgery. Ovaries were removed from the reproductive tracts and washed twice in Transport Media. The ovaries were sliced in Transport Media using a sterile blade to release oocytes. Grade I oocytes as described by Hewitt et al 1998 (darkly pigmented and completely surrounded by one or more layers of cumulus cells) were selected for culture (17).

Experiment 1: progesterone or estrogen oocyte culture

Groups of 30 to 50 grade I oocytes were cultured in 1.5 ml of Basic Culture media. Basic culture media is composed of TCM 199 without phenol red (Sigma, St. Louis, MO) and 100 IU/ml Penicillin (Gibco, Grand Island, NY), 50 μg/ml streptomycin (Gibco), 10 ml/L Na Pyruvate (Gibco), 3 g/L BSA (Fraction V, Fatty acid free Sigma), 5.8 g/L Hepes (Sigma), 100 ml/L L-glutamaine (Sigma). Progesterone (Sigma) or estradiol (Sigma) was added directly to the culture dish containing oocytes to obtain a final concentration of 0.1 μg/ml, 1.0 μg/ml or 10.0 μg/ml. Cultures were incubated at 38.8^{o}C with 5% CO_2 for 24, 48, 72 or 96 hours. After each 24 hour period, one ml of the culture media was removed and immediately replaced with fresh, warmed media with or without hormone supplement. Control oocytes with no hormone supplement were cultured for the same length of time as being examined.

Experiment 2: rFSH oocyte Culture

Experiment two followed the same design as experiment one. Treatment groups were supplemented with recombinant FSH (Serono, Randolph, MA) to reach a final concentration of 0.1 IU/ml, 1.0 IU/ml or 10.0 IU/ml. A control group of oocytes receiving no hormone supplement was cultured for the same length of time as that being examined.

Experiment 3: eCG oocyte Culture

Experiment three followed the same design as experiment one. Treatment groups were supplemented with eCG (Diosynth, Chicago) to obtain a final concentration of 0.1 IU/ml, 1.0 IU/ml or 10.0 IU/ml. Cultures were incubated at 38.8°C with 5% CO_2 for 24, 48, 72 or 96 hours. Oocytes with no hormone supplement were cultured for the same length of time as being examined, to serve as a control.

Experiment 4: LH oocyte Culture

For experiment four we had a limited supply of ovine LH, so groups of 25-30 oocytes were cultured in 50 µl droplets of IVM supplemented with ovine LH (provided by National Hormone and Pituitary Program) to reach final concentrations of 0.1 IU/ml or 1.0 IU/ml. The media droplets were submerged in embryo tested mineral oil (Sigma). A control group of oocytes receiving no hormone supplement was cultured for the same length of time as that being examined. Cultures were incubated at 38.8°C with 5% CO_2 for 96 hours. After each 24 hour period, oocytes were moved to new media droplets containing fresh media with or withour LH supplement.

Experiment 5: L-cysteine oocyte culture

Experiment five followed the same design as experiment one except treatment groups were supplemented with L- Cysteine (Sigma) to obtain a final concentration of at 0.1 mM/ml or 1.0 mM/ml. Cultures were incubated at 38.8^{o}C with 5% CO_2 for 72 hours. After each 24 hour period, 1.0 ml of the culture media was removed and immediately replaced with fresh, warmed media or media containing L-Cysteine supplement.

Experiment 6: Arachidonic acid

Experiment six followed the same design as experiment one except treatment groups were supplemented with arachidonic acid to obtain a final concentration of 10 µM/ml or 100 µM/ml. Cultures were incubated at 38.8^{o}C with 5% CO_2 for 72 hours.

Assessment of Nuclear Maturation

Meiotic maturation was assessed by staining cultured oocytes with the fluorescent dye Hoechst 33342 (Sigma). Prior to staining, removal of cumulus cells to enhance viewing of the oocytes was done by placing oocytes post culture in 1.0 ml of a hyaluronidase solution (500,000 U/L, Specialty Media, Phillipsburg, NJ) for 20 minutes at 38.8^{o}C followed by vortexing on high for 15 minutes. Oocytes were then stained with Hoechst 33342 (Sigma) for 20 minutes and viewed on an inverted microscope equipped with micromanipulators and a fluorescent light source. Stained oocytes were classified as either germinal vesicle (GV) (tight compact nucleus), germinal vesicle break down (GVBD) (nucleus less compact), metaphase I/anaphase I (MI/AI) (presence of a metaphase plate/chromatids separating), metaphase II (MII) (extrusion of a polar body) or unidentifiable (U) (unclassifiable nuclear material).

Statistical Analysis

For all experiments, the various treatments were analyzed and compared to a group cultured in media with no hormone supplement. Statistical analysis was performed by using a multinomial regression model fitted for the data set. The four levels of response were GV, GVBD, MI/AI, MII. SAS procedure Proc Genmode was used to carry out the data analysis. The various treatments were analyzed and compared to a group cultured in media with no hormone supplement. Data was analyzed by proportional odds model using SAS procedure Proc Logistic and Chi-square test.

RESULTS

Experiment 1: Progesterone or estradiol

For Experiment one, a total of 3,421 oocytes were cultured, stained and classified. Classification of oocytes for each culture period and treatment is reported in tables 1 through 4.

There was no effect ($p>0.05$) of treatment, culture time, or treatment and culture time interaction. While MII was the classification of greatest interest, very few of the treatments resulted in MII oocytes. The highest rate of maturation to MII was 5/115 (4.35%) from the 96 hour culture control treatment. The 96 hour culture control treatment also resulted in the highest percentage of MI/AI, 19/115 (16.52%).

Experiment 2: rFSH

Data for experiment two is exhibited in tables five through eight and a total of 1,787 oocytes were cultured . Oocytes cultured in the presence of rFSH were significantly ($p<.05$) more likely to mature to metaphase II than oocytes in the control treatment. The highest rate of maturation to MII was 5/113 (4.42%) from the 96 hour culture 1.0 IU/ml rFSH treatment, although the 0.1 IU/ml rFSH and control treatments also resulted in MII oocytes. While statistical significance was found in the length of culture time ($p<0.05$), with 96 hours being the most likely group to extrude a polar body, no statistical significance was found between the varying concentrations of rFSH examined for this study.

Experiment 3: eCG

A total of 1,384 oocytes were cultured for experiment three(tables nine through twelve). The control treatment was shown to have a significantly ($p<0.05$) greater effect of maturing oocytes to MII when compared to oocytes treated in any of the three concentration of eCG for all of the culture periods. The various concentrations of eCG tested and the culture lengths examined were not statistically significant.

Experiment 4: LH

A total of 426 oocytes were cultured for experiment four (table thirteen). The difference between maturation of LH treated oocytes when compared to nuclear maturation of oocytes cultured without the presence of LH (controls) was noted to be insignificant ($p > 0.05$). No significant nuclear maturation difference ($p> 0.05$) was noted between oocytes cultured in the two concentrations of LH.

Experiment 5: L-cysteine

A total of 1,096 oocytes were cultured for experiment five (table fourteen). Maturation of oocytes tested in all of the L-cysteine supplemented groups was not statistically significant ($p>0.05$) when compared to oocytes cultured in the control media containing no L-cysteine supplement ($p=.29$). The 0.1 mM/ml concentration of L-cysteine resulted in the largest percentage of MI/AI oocytes (2.78%) and MII oocytes (1.85%) of any group including the control group. None of the oocytes treated with the 1.0 μg/ml concentration of L-cysteine resulted in MII oocytes.

Experiment 6: Arachidonic acid

A total of 422 oocytes were cultured for experiment six (table fifteen). Only the control treatment resulted in MII oocytes. The control treatment was significantly more likely ($p<0.05$) to result in MI/AI oocytes when compared to the 10 μM/ml arachidonic acid treatment. There was no statistical significance noted between the control treatment and the 100 μM/ml arachidonic acid treatment when considering likelihood of oocytes maturing to MI/AI.

DISCUSSION

Our objective was to examine the affect of estradiol, progesterone, rFSH, eCG, LH, L-cysteine and arachidonic acid at varying concentrations and hours of culture on the nuclear maturation of canine oocytes.

Experiment one indicated that single hormone supplementation of progesterone and estradiol is not effective for in vitro maturation of canine oocytes. Advanced stages of meiotic maturation, MI/AI and MII, were most likely when oocytes were cultured in vitro

with no progesterone or estradiol supplement. Similar reports by Hewitt and England found no significant difference between in vitro maturation for 48 and 96 hours in a control media and media supplemented with estradiol, progesterone or the two steroid hormones in combination (13).

Experiment two indicated that rFSH supplemented media benefits canine oocytes cultured for 96 hours. For the three concentrations of rFSH examined, no single concentration was found more likely to mature oocyte to MII. This contradicts studies reported by Hewitt and England (16). While they reported 12% (3/25) of oocytes cultured in 1 μg/ml of FSH matured to MI/AI/MII after 96 hours of culture, there was no significant difference between the FSH treatment and the control oocytes.

In experiment three the control treatment was statistically more likely to mature oocytes to MII when compared to either of the eCG treatments. Although this equine hormone is commercially available, it is not likely to be identical any hormone produced by the bitch during estrus and therefore might not be recognized by receptors in the bitch. Similarily, for experiment four we choose to use ovine LH because of its availability as our gonadotropin source. The ovine LH may not have been fully recognized by LH receptors on the canine oocyte therefore not eliciting typical in vivo effects.

While hormone supplementation in vitro has had beneficial effects on oocyte maturation with other species when compared to in vitro culture with no hormone supplements, there are many issues to consider (20,30). One consideration involves the concentration of the hormones used. While serum hormone levels have been reported, the exact hormone concentrations within the oviduct, where the canine oocyte matures to MII, are unknown. In vivo, serum concentration of steroid hormones and gonadotropins fluctuate over time, levels do not remain static as seen in these in vitro studies. Studies using sequential increase and or decrease of a hormone's concentration may provide more information relative to the in vivo situation. Additionally, in vivo, oocytes are exposed to gonadotropins in conjunction with steroid hormones. For these experiments, we choose to examine the effect of

a single gonadotropin or steriod hormone alone although this does not truly mimic the in vivo environment. Future studies incorporating steroid hormones along with endogenous gonadotropins and changes in concentration over time, may improve maturation of canine oocytes in vitro.

Although the L-cysteine treatment groups were not significantly different from the control group for experiment five, it is interesting to note that the 0.1 mM/ml resulted in higher percentages of all four response levels (GV. GVBD, MI/AI, MII) than the control group after 72 hours of culture. It is possible that response of oocytes in this treatment may increase to significant levels if oocytes were examined after 96 hours of culture.

Experiment five did not show L-cysteine supplementation to be beneficial for maturing canine oocytes in vitro. Very little has been reported regarding possible transport mechanisms of cysteine and GSH within the canine oocyte. A lack of gap junctions between the oocyte and cumulus cells may cause disruption in the normal transport of L-cysteine across cell membranes therefore limiting its actions. Our lab did not have the means to measure actual GSH content of the canine oocytes treated in the media containing L-cysteine. This information would be helpful in future experiments to assess the ability of L-cysteine in media to effectively elevate GSH levels in the canine oocyte in vitro.

In contrast to our results, Luvoni et al. 2001 report concluded that "intracellular GSH synthesis can be effectively increased by cysteine-containing media for in vitro maturation of feline oocytes" (26). For their experiment, oocytes were cultured in an IVM media containing 0.5 IU/ml FSH and 0.5 IU/ml LH supplemented with L-cysteine (26). There have been no studies done to test the effects of the FSH and LH alone on GSH production in feline oocytes. Experiment two showed that canine oocytes in vitro matured for 96 hours in basic media containing 1.0 IU/ml FSH, resulted in more advanced stages of nuclear development compared to control oocytes.

In our experiment we did not introduce sperm to the oocyte in culture. L-cysteine has been shown to have a substantial role in decondensation of the sperm head post fertilization

(45). While very little is known concerning GSH production in the canine oocyte matured in vitro at this time, and although no significance was noted in this trial, L-cysteine supplementation in culture of canine oocytes may still prove beneficial for producing canine embryos in vitro, when other supplements have been clearly defined. In future studies, incorporation of sperm or sperm factors may provide possible imformation on any role these may play on resumption of meiosis in oocytes.

The low rates observed in canine oocyte in vitro maturation could be attributed to both inadequate culture conditions and low meiotic competence of oocytes obtained from bitches during anestrus. A recent study that suggests different stages of the estrus cycle may effect the ability of canine oocytes to communicate with cumulus cells indicating a possible link with meiotic competence (25). In mammalian ovaries, gap junctions exist between the somatic compartment of the follicle and the oocyte (25). These gap junctions are involved with the regulation of oocyte meiotic differentiation and maturation, leading to the acquisition of meiotic and developmental competence (25). The Luvoni study suggests that cumulus-oocyte complexes obtained from bitches in anestrus are unable to complete meiotic maturation possibly due to a lack of communications through gap junctions of the germinal and the somatic regions of the oocyte. In contrast, oocytes obtained from bitches in late pro-estrus were able to complete meiosis (25). For oocytes collected during proestrus 89% (16/18) showed open communications through gap junctions while 0% (0/20) of oocytes collected during anestrus showed open communications (25).

The ovaries used to obtain oocytes in the current study are from bitches undergoing routine ovariohysterectomy. These bitches may be in any stage of the estrus cycle but it is more likely that most are in anestrus. We have no available way of knowing the stage of the estrus cycle a bitch is currently in when we obtain her ovaries and oocytes Development of an IVM system for canine oocytes would be most beneficial if applicable to oocytes collected from bitches in all stages of the estrus cycle.

Assisted reproductive techniques are essential tools for aiding in the protection of valuable genetic material, such as gametes of endangered species, along with improving available treatments for infertility cases. Concern for endangered species who are related to the dog and dog owners faced with their pet's infertility have opened a renewed interest in developing ART techniques clinically applicable for the dog. Along with that of others in the field, our study shows the role of steroid hormones, gonadotropins and other factors on canine oocyte maturation to be inconclusive. A clear understanding of the physiology of the immature and mature canine oocyte and the canine oviductal environment may be necessary to develop reliable IVM and IVF techniques for the dog that are applicable to clinical intervention.

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Table 1: Experiment 1 estrogen and progesterone 24 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 µg/ml, Estradiol, 0.1, 1.0 or 10.0 µg/ml Progesterone, or no supplement (control) for 24 hours.

	Estradiol 0.1 µg/ml	Estradiol 1.0 µg/ml	Estradiol 10.0 µg/ml	Progesterone 0.1 µg/ml	Progesterone 1.0 µg/ml	Progesterone 10.0 µg/ml	Control
GV (%)	1/72 (1.39)	11/199 (5.53)	0/97 (0)	0/111 (0)	12/94 (12.77)	0/64 (0)	2/42 (4.76)
GVBD (%)	32/72 (44.44)	92/199 (46.23)	50/97 (51.55)	26/111 (23.42)	29/94 (30.85)	13/64 (20.31)	6/42 (14.29)
MI/AI (%)	0/72 (0)	1/199 (0.50)	0/97 (0)	0/111 (0)	5/94 (5.32)	0/64 (0)	0/42 (0)
MII (%)	0/72 (0)	1/199 (0.50)	0/97 (0)	0/111 (0)	0/94 (0)	0/64 (0)	0/42 (0)
Unknown (%)	39/72 (54.17)	94/199 (47.24)	47/97 (48.45)	85/111 (76.58)	48/94 (51.06)	51/64 (79.69)	34/42 (80.95)

Table 2: Experiment 1 estrogen and progesterone 48 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 µg/ml, Estradiol, 0.1, 1.0 or 10.0 µg/ml Progesterone, or no supplement (control) for 48 hours.

	Estradiol 0.1 µg/ml	Estradiol 1.0 µg/ml	Estradiol 10.0 µg/ml	Progesterone 0.1 µg/ml	Progesterone 1.0 µg/ml	Progesterone 10.0 µg/ml	Control
GV (%)	0/144 (0)	3/124 (2.42)	7/107 (6.54)	0/89 (0)	1/104 (0.96)	0/178 (0)	89/544 (16.36)
GVBD (%)	22/144 (15.28)	67/124 (54.03)	48/107 (44.86)	37/89 (41.57)	67/104 (64.42)	44/178 (2442)	177/544 (32.54)
MI/AI (%)	3/144 (2.08)	5/124 (4.03)	8/107 (7.48)	0/89 (0)	0/104 (0)	0/178 (0)	21/544 (3.86)
MII (%)	0/144 (0)	1/124 (0.81)	0/107 (0)	0/89 (0)	0/104 (0)	0/178 (0)	11/544 (2.02)
Unknown (%)	119/144 (82.64)	48/124 (38.71)	44/107 (41.12)	52/89 (58.43)	36/104 (34.62)	134/178 (75.28)	246/544 (45.22)

Table 3: Experiment 1 estrogen and progesterone 72 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 μg/ml, Estradiol, 0.1, 1.0 or 10.0 μg/ml Progesterone, or no supplement (control) for 72 hours.

	Estradiol 0.1 μg/ml	Estradiol 1.0 μg/ml	Estradiol 10.0 μg/ml	Progesteroe 0.1 μg/ml	Progesterone 1.0 μg/ml	Progesterone 10.0 μg/ml	Control
GV (%)	7/120 (5.83)	5/95 (5.26)	38/94 (40.42)	0/56 (0)	1/84 (1.19)	0/113 (0)	5/94 (5.32)
GVBD (%)	44/120 (36.67)	48/95 (50.53)	35/94 (37.23)	23/56 (41.07)	34/84 (40.48)	50/113 (44.25)	66/94 (70.21)
MI/AI (%)	14/120 (11.67)	10/95 (10.53)	5/94 (5.32)	1/56 (1.79)	6/84 (7.14)	1/113 (0.88)	1/94 (1.06)
MII (%)	3/120 (2.50)	3/95 (3.16)	0/94 (0)	0/56 (0)	0/84 (0)	0/113 (0)	1/94 (1.06)
Unknown (%)	52/120 (43.33)	29/95 (30.53)	16/94 (17.02)	32/56 (57.14)	43/84 (51.19)	62/113 (54.87)	21/94 (22.34)

Table 4: Experiment 1 estrogen and progesterone 96 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 μg/ml, Estradiol, 0.1, 1.0 or 10.0 μg/ml Progesterone, or no supplement (control) for 96 hours.

	Estradiol 0.1 μg/ml	Estradiol 1.0 μg/ml	Estradiol 10.0 μg/ml	Progesterone 0.1 μg/ml	Progesterone 1.0 μg/ml	Progesterone 10.0 μg/ml	Control
GV (%)	3/126 (2.38)	0/185 (0)	4/88 (4.54)	0/79 (0)	0/99 (0)	0/104 (0)	23/115 (21.74)
GVBD (%)	53/126 (42.06)	28/185 (15.13)	48/88 (54.55)	33/79 (41.77)	16/99 (16.16)	38/104 (36.54)	32/115 (27.83)
MI/AI (%)	0/126 (0)	0/185 (0)	0/88 (0)	1/79 (1.27)	3/99 (3.03)	1/104 (0.96)	19/115 (16.52)
MII (%)	0/126 (0)	0/185 (0)	0/88 (0)	0/79 (0)	0/99 (0)	0/104 (0)	5/115 (4.35)
Unknown (%)	70/126 (55.56)	157/185 (84.86)	36/88 (40.91)	45/79 (56.96)	80/99 (80.81)	65/14 (62.50)	34/115 (29.56)

Table 5: Experiment 2 FSH 24 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 IU/ml, rFSH or no supplement (control) for 24 hours.

	rFSH 0.1 IU/ml	rFSH 1.0 IU/ml	rFSH 10.0 IU/ml	Control
GV (%)	4/140 (2.86)	4/97 (4.12)	2/102 (1.96)	4/135 (2.96)
GVBD (%)	30/140 (21.43)	16/97 (16.49)	15/102 (14.71)	51/135 (37.78)
MI/AI (%)	4/140 (2.86)	2/97 (2.06)	3/102 (2.94)	7/135 (5.19)
MII (%)	2/140 (1.43)	0/97 (0)	0/102 (0)	3/135 (2.22)
Unknown	100/140 (71.43)	75/97 (0)	82/102 (80.39)	71/135 (52.59)

Table 6: Experiment 2 FSH 48 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 IU/ml, rFSH or no supplement (control) for 48 hours.rFSH.

	rFSH 0.1 IU/ml	rFSH 1.0 IU/ml	rFSH 10.0 IU/ml	Control
GV (%)	1/86 (1.16)	2/100 (2.00)	0/111 (0)	8/107 (7.48)
GVBD (%)	25/86 (29.07)	35/100 (35.00)	36/111 (32.43)	33/107 (30.84)
MI/AI (%)	5/86 (5.81)	4/100 (4.00)	3/111 (2.70)	9/107 (8.41)
MII (%)	0/86 (0)	0/100 (0)	1/111 (0.90)	1/107 (0.93)
Unknown	61/86 (70.93)	59/100 (59.00)	71/111 (63.96)	56/107 (52.33)

Table 7: Experiment 2 FSH 72 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 IU/ml, rFSH or no supplement (control) for 72 hours.

	rFSH 0.1 IU/ml	rFSH 1.0 IU/ml	rFSH 10.0 IU/ml	Control
GV (%)	5/109 (4.59)	3/118 (2.54)	0/103 (0)	14/115 (12.17)
GVBD (%)	32/109 (29.36)	37/118 (31.36)	37/103 (35.29)	32/115 (27.83)
MI/AI (%)	5/109 (4.59)	4/118 (3.39)	4/103 (3.88)	3/115 (2.61)
MII (%)	0/109 (0)	0/118 (0)	1/103 (0.97)	1/115 (0.87)
Unknown	57/109 (52.29)	74/118 (62.71)	61/103 (59.22)	64/115 (55.65)

Table 8: Experiment 2 FSH 96 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 IU/ml, rFSH or no supplement (control) for 96 hours.

	rFSH 0.1 IU/ml	rFSH 1.0 IU/ml	rFSH 10.0 IU/ml	Control
GV (%)	0/131 (0)	0/113 (0)	0/101 (0)	13/119 (10.92)
GVBD (%)	21/31 (16.03)	16/113 (14.16)	15/101 (14.85)	24/119 (20.17)
MI/AI (%)	4/131 (3.05)	4/113 (3.54)	6/101 (5.94)	2/119 (1.68)
MII (%)	1/131 (0.76)	5/113 (4.42)	0/101 (0)	0/119 (0)
Unknown	105/131 (80.15)	88/113 (77.88)	80/101 (79.21)	80/119 (67.23)

Table 9: Experiment 3 eCG 24 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 IU/ml, eCG or no supplement (control) for 24 hours.

	eCG 0.1 IU/ml	eCG 1.0 IU/ml	eCG 10.0 IU/ml	Control
GV (%)	13/260 (6.15)	0/41 (0)	0/115 (0)	0/105 (0)
GVBD (%)	52/260 (20.00)	6/41 (14.63)	33/115 (28.70)	38/105 (36.19)
MI/AI (%)	1/260 (0.38)	0/41 (0)	27/115 (23.48)	7/105 (6.67)
MII (%)	0/260 (0)	0/41 (0)	0/115 (0)	2/105 (1.90)
Unknown	191/260 (73.46)	35/41 (85.37)	55/115 (47.83)	58/105 (55.24)

Table 10: Experiment 3 eCG 48 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 IU/ml, eCG or no supplement (control) for 48hours.

	eCG 0.1 IU/ml	eCG 1.0 IU/ml	eCG 10.0 IU/ml	Control
GV (%)	2/57 (3.51)	1/50 (0.20)	6/90 (6.67)	0/42 (0)
GVBD (%)	23/57 (40.35)	29/50 (58.00)	34/90 (37.78)	31/42 (73.81)
MI/AI (%)	1/57 (1.75)	0/50 (0)	0/90 (0)	0/42 (0)
MII (%)	0/57 (0)	0/50 (0)	0/90 (0)	0/42 (0)
Unknown	31/57 (54.39)	20/50 (40.00)	50/90 (55.56)	11/42 (26.19)

Table11: Experiment 3 eCG 72 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 IU/ml, eCG or no supplement (control) for 72hours.

	eCG 0.1 IU/ml	eCG 1.0 IU/ml	eCG 10.0 IU/ml	Control
GV (%)	0/43 (0)	0/44 (0)	3/115 (2.61)	0/35 (0)
GVBD (%)	22/43 (51.16)	23/44 (52.27)	23/115 (20.00)	18/35 (51.43)
MI/AI (%)	2/43 (4.65)	0/44 (0)	0/115 (0)	1/35 (2.86)
MII (%)	0/43 (4.65)	1/44 (2.27)	0/115 (0)	0/35 (0)
Unknown	19/43 (44.19)	20/44 (45.45)	89/115 (77.39)	16/35 (45.71)

Table 12: Experiment 3 eCG 96 hour culture data collected from oocytes cultured with 0.1, 1.0 or 10.0 IU/ml, eCG or no supplement (control) for 96hours.

	eCG 0.1 IU/ml	eCG 1.0 IU/ml	eCG 10.0 IU/ml	Control
GV (%)	4/195 (2.05)	16/45 (35.56)	0/120 (0)	0/27 (0)
GVBD (%)	91/195 (46.67)	19/45 (42.22)	12/120 (10.08)	4/27 (14.81)
MI/AI (%)	15/195 (7.69)	2/45 (4.44)	3/120 (2.50)	3/27 (11.11)
MII (%)	0/195 (0)	0/45 (0)	0/120 (0)	1/27 (3.70)
Unknown	85/195 (43.59)	8/45 (17.78)	105/120 (87.50)	19/27 (70.37)

Table 13: Experiment 4 LH 96 hour culture data collected from oocytes cultured with 0.1 or 1.0 IU/ml LH or no supplement (control) for 96 hours.

	LH 0.1 IU/ml	LH 1.0 IU/ml	Control
GV (%)	5/142 (3.52)	8/128 (6.25)	3/156 (1.92)
GVBD (%)	54/142 (38.03)	49/128 (38.28)	40/156 (25.64)
MI/AI (%)	14/142 9.86)	10/128 (7.81)	6/156 (3.85)
MII (%)	2/142 (1.41)	0/128 (0)	0/156 (0)
Unknown (%)	67/142 (47.18)	61/128 (47.65)	107/156 (68.59)

Table 14: Experiment 5 L-cysteine 96 hour culture data collected from oocytes cultured with 0.1 or 1.0 mM/ml, L-cysteine or no supplement (control) for 72hours.

	L- cysteine 0.1 mM/ml	L-cysteine 1.0 mM/ml	Control
GV (%)	1/432 (0.23)	4/384 (1.04)	0/280 (0)
GVBD (%)	196/432 (45.37)	139/384 (36.20)	95/280 (0)
MI/AI (%)	12/432 (2.78)	7/384 (1.82)	4/280 (1.43)
MII (%)	8/432 (1.85)	0/384 (0)	2/280 (0.71)
Unknown (%)	215/432 (49.77)	234/384 (0)	179/280 (63.93)

Table 15: Experiment 6 Arachidonic acid 72 hour culture data collected from oocytes cultured with 10 or 100 mM/ml, Arachidonic Acid or no supplement (control) for 72hours.

	Arachidonic Acid 10 mM/ml	Arachidonic Acid 100 mM/ml	Control
GV (%)	0/121 (0)	0/121 (0)	0/180 (0)
GVBD (%)	46/121 (38.02)	10/121 (8.26)	58/180 (32.22)
MI/AI (%)	4/121 (0.033)	9/121 (7.44)	23/180 (12.78)
MII (%)	0/121 (0)	0/121 (0)	1/180 (0.56)
Unknown (%)	71/121 (58.76)	102/121 (84.30)	98/180 (54.44)

CHAPTER III

CONCLUSION

Assisted reproductive technologies have been developed for use in a variety of mammal. Specifically. now they are desired as clinical alternatives by dog owners facing infertility in their companion animals. These technologies including in vitro fertilization and intracytoplasmic sperm injection require the use of a metaphase II oocyte. The bitch ovulates an immature oocytes which matures to metaphase II while in the oviduct two days after ovulation. Oocyte in vitro maturation protocols used successfully in other species have failed to reliably mature canine oocytes to metaphase II.

We examined over 8,536 canine oocytes for nuclear maturation, analyzing the effects of a basic culture media supplemented with hormones (estradiol, progesterone, FSH, eCG, LH), the antioxidant L-cysteine or arachidonic acid. In each case an individual treatment was compared to basic media containing no supplements. The FSH treatments results in significantly ($p < 0.05$) more oocytes reaching metaphase II than the control group. None of the other hormones, the antioxidant, or arachidonic acid were shown to produce significantly more mature oocytes than the control group.

Our findings, along with that of other investigators, suggest that there are physiologic differences in the canine reproductive tract and the canine oocyte compared to other mammals. Succesfful maturation strategies may require innovative technologies or a different order of supplements. But the addition of rFSH appears to support the maturation of canine preantral oocytes to metaphase II.