THESIS

ASSOCIATION OF OOCYTE AND EARLY EMBRYO MORPHOLOGY WITH AGE AND THE ESTABLISHMENT AND MAINTENANCE OF PREGNANCY AFTER ICSI IN MARES

Submitted by

Bethany Linda Frank-Guest

Department of Biomedical Sciences

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY BETHANY LINDA FRANK-GUEST, ENTITLED "ASSOCIATION OF OOCYTE AND EARLY EMBRYO MORPHOLOGY WITH AGE AND THE ESTABLISHMENT AND MAINTENANCE OF PREGNANCY AFTER ICSI IN MARES" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate work

George Seidel Jr.

Dean Hendrickson

Advisor: Elaine Carnevale

Department Head: Barbara Sanborn

ABSTRACT OF THESIS

ASSOCIATION OF OOCYTE AND EARLY EMBRYO MORPHOLOGY WITH AGE AND THE ESTABLISHMENT AND MAINTENANCE OF PREGNANCY AFTER ICSI IN MARES

Increasing maternal age in humans, horses and lab animals has been associated with a decrease in fertility. Oocyte quality and morphology have been implicated as primary causes of reduced fertility in older mares. Selected oocyte morphological parameters have been correlated with pregnancy development in humans and horses. Objective measurements of morphology to assess oocyte quality would provide a critical evaluation and help identify zygotes with the highest developmental potential for transfer, to optimize recipient utilization and pregnancy rates.

The hypotheses of the research were that oocyte and early embryo morphology differ with donor mare age and correspond with developmental potential. Objectives for the first study were to compare: 1) oocyte donor age with oocyte morphology and developmental competency after ICSI, and 2) oocyte morphology with developmental competency (cleavage, early pregnancy, late pregnancy and pregnancy loss) after ICSI. Objectives for the second study were to compare developmental potential of ICSI-produced embryos with: 1) oocyte donor age, and 2) cleavage characteristics, and 3) rate of embryonic development.

Oocytes were collected from donor mares in a clinical ICSI programs. The mares were divided into the following age groups and fertility categories: 1) 3-13 yr with Known fertility, 2) 2-13 yr with Unknown fertility, 14-19 yr, 20-23 yr and 24-27 yr. Approximately 24 h after induction of follicle maturation, and oocytes were collected and cultured approximately 18 h before being stripped of cumulus cells. Photographic images (200x) were captured before oocytes were injected with sperm. Images of oocytes were measured using digital calipers within a computer software program.

Ooplasm volume was larger ($p<0.05$) for oocytes from mares 14-19 yr and 20-23 yr than mares 3-13 yr Known than for mares 24-27 yr. Perivitelline space volume was similar between mares 3-13 yr Unknown and mares 20-23 yr, but was smaller ($p<0.05$) between mares 3-13 Unknown and the other age groups. Oocyte diameter (OD) was smaller ($p=0.05$) between oocytes from donors 3-13 yr Known and donors 14 -19 yr, but similar among all other groups. Inner zona pellucida diameter (IZPD) differed ($p=0.03$) only between mares 14-19 yr and mares 3-13 yr Unknown, with oocytes from mares 14-19 yr having the largest numerical IZPD and mares 3-13 yr Unknown having the smallest IZPD. Ooplasm diameter (OpD) was smaller ($p\leq0.02$) for oocytes from mares 3-13 yr Known than from mares 14-19 or 20-23 yr. The diameter of the zona pellucida with the surrounding matrix (ZPTM) was greater ($p<0.05$) for mares 3-13 yr Unknown than for all other groups.

The rate of embryo development (hours per cell) prior to oviductal embryo transfers was faster ($P<0.05$) for embryos that did versus did not produce an early pregnancy and tended ($P\leq0.1$) to be faster for embryos that did versus did not produce a

late pregnancy. Embryonic vesicles that had a more rapid increase in diameter were more often ($p<0.05$) maintained to the late pregnancy stage.

Donor mare age exerted a large effect on the development and outcome of pregnancies. Oocyte morphology was not a reliable indicator of oocyte developmental potential, although speed of early embryonic development was associated with embryonic competency.

Bethany Linda Frank-Guest
Department of Biomedical Sciences
Colorado State University
Fort Collins, CO 80523
Spring 2010

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CHAPTER 1.0

Introduction

Oocyte quality and morphology have been implicated as primary causes of reduced fertility in older mammals (Carnevale et al., 1995, Krisher, 2004). Increasing maternal age in humans, horses and lab animals has been associated with a decrease in fertility (Talbert, 1968). Oocyte numbers decrease with advancing age; however, a decline in fertility occurs before complete depletion of the ovarian oocyte supply. The aged mare is a predominant focus in the equine industry, with preservation of valuable genetics a critical goal for many producers and scientists. Many mares prove their genetic potential only in later years when fertility has already begun to decline. The age of the donor, whether human or equine, contributes significantly to the quality of oocytes and the development of the subsequent zygote.

Oocyte transfer was used to study differences in viability of oocytes from young (6 to 10 yr) and old mares (20 to 26 yr) (Carnevale et al., 1995). Pregnancy rates were significantly higher for oocytes from young mares (92%) transferred into young recipients versus oocytes from aged mares (31%) transferred into young recipients. In vitro fertilization is used successfully in humans and cattle, however, with limited success in the horse. Only two foals have been born from in vitro fertilization of in vivo matured oocytes (Palmer et al., 1991). Approximately 15% of women of childbearing age in the

United States have received an infertility treatment, and 69% of in vitro fertilization cycles do not result in pregnancy (Krisher, 2004). Due to the inconsistency of in vitro fertilization, alternative methods have been identified for assisted fertilization in the horse such as intracytoplasmic sperm injection (ICSI). In preparation for ICSI, the oocyte is stripped of surrounding cumulus cells, and the oocyte can be observed for morphologic evaluation and assessment. This assessment provides information about potential viability of the oocyte and age-associated changes in oocyte morphology. The first pregnancy from an in vitro matured equine oocyte was reported in 1996 (Squires et al., 1996). Results for ICSI remained relatively inconsistent until the Piezo drill was used. With the utilization of the Piezo drill, high fertilization and cleavage rates (>80 % cleavage) have been obtained (Choi et al., 2002, Galli et al., 2002). Failure to obtain pregnancies in both humans and horses may be the result of defective oocytes.

Substantial information regarding oocyte morphology has been obtained from oocytes before ICSI procedures. Many morphological parameters of the oocyte can be assessed such as shape, cytoplasm color and granulation, regularity and thickness of the zona pellucida, size of the perivitelline space, presence of vacuoles, presence or absence of germinative vesicles, and polar body appearance (Navarro et al., 2009, Xia, 1997). Oocyte morphology has been more thoroughly evaluated in humans than horses due to the availability of oocytes. Early embryo morphology has been evaluated critically in humans before transfer after in vitro procedures. Late embryo morphology is routinely evaluated in most domestic species.

Two studies were done using retrospective data from the clinical Assisted Reproductive Program at Colorado State University. In the first, we investigated the hypothesis that oocyte morphology would differ with age and would correspond with developmental potential. Objectives were to compare: 1) oocyte donor age with oocyte morphology and developmental competency after ICSI, and 2) oocyte morphology with developmental competency (cleavage, early pregnancy, late pregnancy and pregnancy loss) after ICSI.

The second study was designed to investigate the hypothesis that early embryo morphology would differ with age and would correspond with developmental potential. Objectives for the second study were to compare developmental potential of ICSI-produced embryos with: 1) oocyte donor age, and 2) cleavage characteristics, and 3) rate of embryonic development.

CHAPTER 2.0

Review of Literature

2.1 Mare Infertility

Aging results in multiple changes to a physiological system. Increasing maternal age in humans, horses and lab animals has been associated with a decrease in fertility (Talbert, 1968). Oocyte numbers decrease with advancing age; however, a decline in fertility occurs before complete depletion of the ovarian oocyte supply. The decline in reproductive capacity is due to hypothalamic-pituitary abnormalities, ovarian endocrine deficiencies, failure of ovulation, decreases in fertilization and implantation rates, impaired oviductal function, decreased endometrial receptivity, and an increase in post implantation embryonic death (Werner et al., 1991, Brinsko et al., 1994, Carnevale et al., 1993, Fugo and Butcher, 1971).

The aged mare is a predominant focus in the equine industry, with preservation of valuable genetics a critical goal for many producers and scientists. Many mares prove their genetic potential in later years when fertility has already begun to decline. The age of the donor, whether human or equine, contributes significantly to the quality of oocytes and the development of the subsequent zygote. The use of in vitro methods for embryo production for the human infertility and the need to enhance genetic gain in economically important livestock species has highlighted the importance of oocyte competence as a

limiting factor in embryo development (Armstrong, 2000). Given the expense and limited availability of oocytes, maximizing the potential of each oocyte is essential. If the capability were available to accurately assess fertility and developmental competence of an oocyte and the subsequent embryo, better selection could be made regarding potential oocyte fate. With humans, fewer multiple births would occur if presumptive zygotes of positive developmental capacity could be identified.

An increase in abnormal cycles and altered follicular development occur with aging in the mare. The length of the follicular phase is associated with the size of the largest follicle at the end of the luteal phase (Carnevale et al., 1993). The growth rate of the ovulatory follicle was faster in mares 5 to 7 yr than in mares 15 yr or older (Carnevale et al., 1993). Mares $\geq$ 20 yr have an increased duration of the follicular phase, follicles of a smaller diameter at luteolysis (Carnevale et al., 1993) and fewer follicles present in each follicular wave than mares 5 to 7 yr (Ginther et al., 1993).When aged mares cycle, they have a delayed entry into the breeding season by 2 weeks (Vanderwall and Woods, 1990), resulting in a 10% loss of the breeding season. During 60 days of observation throughout the breeding season, only 50% of mares older than 20 yr had normal estrous cycles, and another 19% did not ovulate or develop follicles larger than 5 mm in diameter (Carnevale, 1994).

Aging effects have been identified in follicular activity and concentrations of hormones in both horses and humans. Greater follicle stimulating hormones (FSH) concentrations led to greater follicular activity during late luteal phase and, therefore, to a shorter follicular phase. Mares older than 20 yr have a longer follicular phase and a less

pronounced luteinizing hormone (LH) surge than mares 5 to 7 yr and 15 to 20 yr (Carnevale et al., 1993). Older mares that fail to develop follicles and ovulate have elevated levels of FSH and LH during period of ovarian inactivity (Carnevale et al., 1994). Ovarian production of inhibin levels increases, and estradiol levels increase with follicle development, which suppresses FSH levels, thereby reducing subsequent follicle numbers (Ginther et al., 2001). The relationship between inhibin, estradiol and FSH is similar in cattle, women and mares, with the exception of a longer interval in mares between increased estrogen and follicle deviation (Ginther et al., 2001). In the mare, estradiol begins to increase the day before the beginning of follicular deviation, thus not contributing to the decline of FSH until the day before deviation (Gastal et al., 1999).

Ovulatory dysfunction can induce subfertility by decreasing the number of viable estrous cycles available for breeding, requiring longer breeding seasons to compensate for delays in initiation or failure of ovulation (Vanderwall and Woods, 1990). The incidence of ovulatory failure is relatively high in the mare, occurring in approximately 8% of cycles (McCue and Squires, 2002). The interval between ovulations was significantly longer for mares 20 to 26 yr versus 10 to 11 yr (Vanderwall et al., 1989).

Oocyte transfer was used to study the difference in viability of oocytes from young (6 to 10 yr) and old (20 to 26 yr) mares (Carnevale et al., 1995). Pregnancy rates were significantly higher for oocytes from young mares transferred into young recipients (92%) versus oocytes from aged mares transferred into young recipients (31%). When the same young and old donor mares were artificially inseminated, the pregnancy rates were 83% and 19%, respectively. The transfer of oocytes from old donors to young recipients

did not eliminate age-associated subfertility, demonstrating that oocytes from old mares are defective. Results indicated that defective oocytes account for most of the subfertility in old mares independent of the effects of oviductal and uterine pathology (Carnevale et al., 1995).

Failure to recover oocytes or embryos from the oviducts of old mares may indicate failure of oocyte discharge, pickup or transport to the site of fertilization (Carnevale et al., 1993). The incidence of oviductal pathology is unknown; however, mares older than 10 yr have been reported to have an increased prevalence of infundibular adhesions (Henry et al., 1981). Oviductal pathology could impair ovulation, sperm transport, fertilization or early embryo transport. The effect of oviductal environment has not been thoroughly evaluated to identify aging effects and subsequent changes in embryo quality. Co-culturing embryos with oviductal cells provides a consistent quality culture system. Day 2 embryos co-cultured with either young or old oviductal cells provides evidence that maternal age affects quality score, cell number and embryo diameter (Brinsko et al., 1994). No differences were noted with young and aged mare embryos when co-cultured with young mare oviductal cells. Quality scores of young mares' embryos co-cultured with aged mare oviductal cells were superior to aged mare embryos co-cultured with aged mare oviductal cells. There were more cells in the embryos co-cultured in young than aged mare oviductal cells, suggesting an effect of the aged mare's oviductal cells (Brinsko et al., 1994).

Age-associated subfertility has been evaluated before entry of zygotes into the uterus, providing information regarding oocyte quality, cleavage capability and oviductal

environment. To assess fertility before entry of embryos into the uterus, embryos and uncleaved ova were harvested from the oviducts of inseminated mares at 24 to 48 h after ovulation (Carnevale et al., 1993). Significantly more embryos (≥ 2 cells) or recently ovulated ova were collected from young mares (3.5 to 7 yr) than old mares (>20 yr). Fewer ova from old mares than young mares were cleaved by day 1.5, indicating an age-associated decline in number of embryos present in oviducts. On day 3, harvested embryos from young versus old mares had more cells (≥ 8 versus 2 to 8, respectively). Embryos from the older mares had higher (poorer) morphology grades, than embryos from young mares, suggesting reduced viability. Most common morphologic changes included large amounts of extracellular debris, granular or dark cytoplasm and cells of different sizes (Carnevale et al., 1993).

With advancing age in mares, uterine functional and histological changes are identified (Carnevale and Ginther, 1992), which can lead to an inadequate uterine environment for embryo development and subsequent loss. Mares older than 15 yr have more extensive intrauterine fluid accumulations, more inflammatory cell infiltrations and more fibrotic changes on uterine biopsy than mares 5 to 7 yr. Functional changes, including delayed embryo fixation and observation of an embryo proper, tend to occur in older mares (Carnevale and Ginther, 1992).

Under breeding farm conditions, mares 20 to 26 yr required 4.6 cycles to conceive and deliver a foal versus two cycles for mares 10 to 11 yr (Vanderwall et al., 1989). Pregnancy rates per cycle decline with advancing age from approximately 60% in younger mares (5 to 7 yr) to 30% in older mares (≥15 yr) (Vanderwall et al., 1989,

Ginther, 1992). Pregnancy rates are on average 28% lower for mares greater than 13 yr of age than for mares 2 to 11 yr of age (Vanderwall and Woods, 1990). Experimentally, pregnancy rates on day 11 were significantly higher for younger mares (3.5 to 7 yr) than for older mares (>20 yr). Mean embryonic vesicle size was significantly larger on day 12 and 13 for young versus old mares (Carnevale et al., 1993). Undersized embryonic vesicles have been associated with reduced embryo viability and an increased incidence of embryo loss (Ginther et al., 1985). Pregnancy rates were significantly higher in young mares (average age of 4.9 yr) than subfertile mares (average age of 17.4 yr) at day 14, despite similar fertilization rates in both groups (Ball et al., 1986). Pregnancy rates were determined on day 14 by transrectal ultrasonography and compared to early pregnancies (day 2 or day 4), which were determined by the recovery of oviductal embryos (Ball et al., 1986, Ball et al., 1989). Pregnancy rates were significantly higher for normal mares on day 14 than for subfertile mares. There was no difference in pregnancy rates between day 2 and day 14 in normal mares; however, in subfertile mares, significantly fewer mares were pregnant at day 14 than on day 2. Similar results were obtained when comparing day 4 pregnancy results to day 14 (Ball et al., 1989).

Early embryonic loss is a result of failure in development occurring due to poor quality oocytes or exposure to inadequate environment (Ball et al., 1986). Embryonic loss rates of 20-30% have been detected in mares greater than 18 yr of age (Vanderwall, 2008) and are determined to be a major cause of subfertility in the aged mare. Aged mares have a higher incidence of early embryonic loss between the days 15 and 50 versus young mares (39% versus 17% loss, respectively) (Villahoz et al., 1985). Fertilization

failure represents 8-19% of fertility losses in subfertile mares, while embryonic loss represents a larger proportion of fertility losses (Ball et al., 1989).

2.2 Human Infertility

The decision to have children later in life for women has brought the impact of age on fertility to the forefront of many research programs. Spontaneous abortions occurred in 9.5% of women 20 to 24 yr and 33.8% of women 40 to 44 yr (Werner et al., 1991). Approximately 15% of women of childbearing age in the United States have received an infertility treatment, and 69% of in vitro fertilization cycles do not result in pregnancy (Krisher, 2004). When pregnancies do result from infertility treatments, multiple conceptuses remain one of the major problems associated with human-assisted conception (Schnorr and Jones, 2001). The pregnancy rate-to-embryo transfer ratio was 20.3%, 9.8% and 9.5% for women under 30, 31 to 35 and over 36 yr, respectively (Werner et al., 1991). After hormonal replacement and oocyte donation, women between 30 and 59 yr of age showed no decrease in pregnancy outcomes (~40%) (Sauer, 1998, Krisher, 2004). The human endometrium retains the ability to respond to gonadal steroids and provides a receptive environment for embryo implantation and gestation even in older women (Werner et al., 1991). Oocyte donation from younger women resulted in pregnancy outcomes in recipient women $\geq$ 40 yr (34-40%) similar to recipient women < 40 yr (30-32%); however, when women $\geq$ 40 yr underwent in vitro fertilization using their own oocytes, pregnancy rates were much lower (0-9%) (Sauer, 1998, Krisher, 2004).

2.3 Assisted Reproductive Procedures in the Horse

Techniques for in vitro fertilization in the horse have lagged behind some other domestic species, primarily due to low availability of oocytes and lack of success of in vitro fertilization. Assisted reproductive technologies in the horse have included embryo transfer, oocyte transfer and, more recently, intracytoplasmic sperm injection (ICSI) and nuclear transfer (cloning).

2.3.1 Oocyte Recovery: In vivo

In the mare, most oocytes are harvested from preovulatory follicles 20 to 36 h after administration of a follicle and oocyte maturation agent such as human chorionic gonadotropin (hCG) or a gonadotropin releasing hormone (GnRH) analogue (deslorelin acetate) (Carnevale and Maclellan, 2006). The two most common methods to harvest oocytes in vivo are by transvaginal, ultrasound-guided follicle aspiration and by transcutaneous flank aspiration. Most commonly, oocytes are harvested via a transvaginal, ultrasound-guided needle attached to a suction pump (Carnevale and Maclellan, 2006). This method allows for repeated, minimally invasive harvesting of oocytes. During the aspiration, the follicle is flushed with sterile, warmed medium, with heparin added to prevent blood clots (Carnevale and Maclellan, 2006).

When oocytes are collected from ovaries from mares not treated with exogenous hormones, a variable recovery rate between 19-35% of oocytes per follicle aspirated is expected (Cook et al., 1993, Alm et al., 1997). Oocyte recovery rates are increased as follicle size increases in diameter to greater than 25 mm, but rates decrease again for follicles greater than 35 mm (Meintjes et al., 1995). A similar recovery method is used

for cattle and horses; however, superior success (55%) and repeatability is achieved in cattle (Pieterse et al., 1991). This is most likely due to the area of attachment of the oocyte in the cow versus mare follicle. The cow has a narrow cumulus attachment, whereas the mare has a broad-base cumulus attachment. The mare's oocyte is imbedded in a thecal pad, requiring more manipulation and vigorous flushing to facilitate oocyte recovery (Hawley et al., 1995, Galli et al., 2007). This method offers a repeatable, less invasive and less traumatic method to obtain equine oocytes than surgical removal of the ovary (Duchamp et al., 1995).

2.3.2 Oocyte Transfer (OT)

Oocytes often are harvested from the preovulatory follicle of a donor mare after exogenous gonadotropin stimulation for follicle and oocyte maturation. The oocyte is transferred surgically via flank laparotomy into the oviduct of an inseminated recipient in which fertilization and embryo development will occur. High pregnancy rates (75-80%) are obtained utilizing oocyte transfer except in aged donors with potentially compromised oocytes (Carnevale and Ginther, 1995, Carnevale et al., 2005). Oocyte transfer has the advantage over traditional embryo transfer in that donor mares are never inseminated; therefore, mares with a history of endometritis or chronic uterine fluid could be well suited for oocyte transfer. Donor mares with oviductal blockage, failure to ovulate and poor embryo flush rates are candidates for oocyte transfer. Recipient mares require extensive management. Oocyte transfer recipients are at risk for post-mating endometritis because they are inseminated. If an oocyte transfer mare were to ovulate her own oocyte,

there would be a risk of her becoming pregnant. Therefore, recipients' oocytes are removed by follicular aspiration, or noncyclic, hormone-manipulated mares are selected.

2.3.3 In Vitro Fertilization (IVF)

In vitro fertilization is commonly used in the human, bovine, and other species; however, in vitro fertilization fails to provide consistent results in the equine. Only two foals have been born from in vitro fertilization of in vivo matured oocytes (Palmer et al., 1991). The failure of in vitro fertilization in the horse may be due to inefficient sperm capacitation (Alm et al., 2001), failure of sperm penetration of the zona pellucida (Hinrichs et al., 2002, Kölle et al., 2007), or incomplete in vitro maturation (Li et al., 2001).

In vitro fertilization is used in the human with moderate success. Embryos produced by in vitro fertilization in humans have a low implantation potential (10-20%) (De Vos and Van Steirteghem, 2000, Sun et al., 2005). Implantation failure in humans may be a result of aneuploidy or an inability of the embryo to escape from the zona pellucida by a means of hatching. The zona pellucida may become excessively hardened due to prolonged or suboptimal culture conditions that may interfere or prevent the natural hatching process (Cohen et al., 1990). In the human, the zona pellucida thins as the blastocyst expands in order for the embryo to prepare for implantation in the uterus. If the zona pellucida fails to break, the blastocyst will be unable to continue expansion. Assisted hatching has been researched in humans to facilitate higher implantation rates; the three most common methodologies utilized include mechanical incision, puntate

acidification, and laser cautery. Laser cautery has proven to be the fastest and most accurate method to date (Sun et al., 2005).

2.3.4 Intracytoplasmic Sperm Injection (ICSI)

Due to the inconsistency of in vitro fertilization, alternative methods are used for assisted fertilization in the horse. Currently, ICSI is being utilized as a method to assist fertilization and facilitate production of valuable foals. In preparation for ICSI, the oocyte is stripped of surrounding cumulus cells, and the oocyte can be observed for morphologic evaluation and assessment. This assessment provides information as to the potential viability of the oocyte and age-associated changes in oocyte morphology. Intracytoplasmic sperm injection requires a mature oocyte in metaphase II, which can be obtained either ex vivo by aspirating a preovulatory follicle of a mare after gonadotropin stimulation or by in vitro maturation of oocytes collected from small immature follicles. An oocyte is enzymatically and mechanically denuded from the surrounding cumulus cells and manipulated using a micromanipulator. A single sperm is picked up using a micropipette and immobilized by breaking the sperm midpiece membrane, releasing sperm cytosolic factors important to oocyte activation. The single sperm is injected into the cytoplasm of the mature oocyte. The use of ICSI circumvents the need for sperm to penetrate the equine zona pellucida. The first pregnancy from an in vitro matured equine oocyte was reported in 1996 (Squires et al., 1996). Results for ICSI remained relatively inconsistent until the Piezo drill was used. With the utilization of the Piezo drill, high fertilization and cleavage rates (>80 % cleavage) have been obtained (Choi et al., 2002,

Galli et al., 2002). Several foals have been born after the transfer of ICSI derived, in vitro cultured equine blastocysts (Li et al., 2001, Galli et al., 2007, Hinrichs et al., 2007).

Stallions with poor-to-no sperm motility, limited sperm concentrations, epididymal collections, or limited sperm availability can be utilized with ICSI. No difference was noted in cleavage or embryo development among oocytes injected with sperm from stallions or men with good, poor or no fertility when motile sperm were selected for ICSI (Lazarri et al., 2002, Choi et al., 2002, Serhal et al., 1997). Embryos derived from ICSI are either surgically transferred into the oviduct as early embryos, at 2 to 5 cells (Cochran et al., 1998), or cultured to the blastocyst stage and transferred transcervically into the uterus (Li et al., 2001, Hinrichs et al., 2005). Lower pregnancy rates have been reported for embryos after culture for even 26 to 28 h after ICSI than after transfer of presumptive zygotes at 4 to 8 h after ICSI (McKinnon et al., 2000).

Recipient mare management differs for ICSI recipients compared to oocyte transfer recipients; the recipients can ovulate their own oocyte, develop a corpus luteum and maintain an endogenous source of progesterone.

2.4 The Oocyte

Most mammals develop their entire set of primordial germ cells in utero. These germ cells undergo meiosis until prophase, at which time they will remain arrested until further development stimulates cytoplasmic, nuclear and molecular maturation. Primary oocytes are surrounded by a single squamous layer of pregranulosa cells and arrested at the diplotene stage of the first meiotic prophase (Meldrum, 1993).

Oocytes acquire developmental competence during follicular growth, reaching meiotic competence at early antral stages of follicle growth. As the oocyte and follicle grow and mature, the oocyte acquires the ability to resume and complete meiosis, successfully undergo the fertilization process, and initiate and sustain embryonic development (First et al., 1988). Many genes involved in meiotic progression and cell cycle control are expressed as the oocyte grows, as well as many other genes (Wickramasinghe and Albertini, 1993). During the later stages of growth, cytoplasmic factors, including mRNA and protein molecules are accumulated and are necessary for embryo development.

Developmental, meiotic and cytoplasmic competences are closely correlated with follicle size (Marchal et al., 2002, Armstrong, 2000). Smaller follicles appear not to be able to complete cytoplasmic maturation. Bovine oocytes smaller than 95 μm in diameter are unable to resume meiosis in vitro, acquiring meiotic competence between 95 and 100 μm. Oocytes of 95 to 104 μm in diameter are capable of fertilization in vitro but have a limited ability to develop to the morula stage. With increasing growth, their ability to develop to the blastocyst stage in vitro gradually increases until they reach optimum rates at maximum diameter of 135 μm (Fair et al., 1995).

Full oocyte competence is acquired during the final stages of preovulatory follicle growth under the influence of a surge of gonadotrophic hormones (FSH and LH) from the pituitary; this surge induces the final stages of cytoplasmic maturation and the oocyte capacitation necessary for full development to the ovulated, meiotically mature egg (Armstrong, 2000). Equine oocyte maturation is associated with well-defined

cytoplasmic and nuclear changes that are paralleled with increased follicular fluid progesterone concentrations and consistently high estrogen concentrations (Kenney et al., 1979). During maturation, the oocyte nucleus resumes meiosis to metaphase II, and cytoplasmic changes occur including migration of cortical granules to the ooplasm periphery.

Assisted reproductive technologies for most species involve the administration of gonadotrophin releasing hormone (GnRH), or a follicle and oocyte maturation agent such as hCG. These exogenous agents help stimulate oocyte maturation and synchronize oocyte recovery from the donor. The administration of exogenous agents enables recovery of oocytes with a higher capacity to develop to the blastocyst stage after fertilization and culture in vitro, as well as providing a more homogeneous population of metaphase II oocytes (Bordignon et al., 1997). Exogenous stimulation may cause several anomalies such as endocrine imbalance, changes in follicular steroidogenesis, abnormal cytoplasmic and nuclear maturation of oocytes, asynchronous follicular growth, and asynchronous ovulation (Bordignon et al., 1997). Abnormal LH profiles have been correlated with several aspects of oocyte quality, including precocious resumption of meiosis, blockage of meiotic maturation metaphase I and asynchronies between cumulus cells and oocyte and between the nuclear and cytoplasmic components of oocytes during maturation (de Loos et al., 1989). Asynchronous nuclear and cytoplasmic maturation may occur when gonadotrophin releasing hormones are administered (Xia, 1997). The effects of exogenous stimulation may be mediated via a loss of direct interaction between the oocyte and cumulus cells (Combelles and Albertini, 2003). Cumulus cell expansion

causes retraction of processes on corona cells that communicate with the oocyte via gap junctions, causing a breakdown of cell-to-cell communication between cumulus cells and oocyte (Armstrong, 2000). Before aspiration, aging of metaphase II oocytes in vivo may be associated with degeneration of the first polar body and result in over maturity of oocytes at fertilization (Xia, 1997).

2.4.1 Oocyte Morphology

Oocyte quality and morphology has been implicated as a primary cause of reduced fertility in older mammals (Carnevale et al., 1995, Krisher, 2004). However, oocyte quality is difficult to assess. Many subjective methods have been evaluated to assess oocyte quality; however, few objective methods have been thoroughly evaluated. Substantial information regarding oocyte morphology has been obtained during ICSI procedures. Prior to ICSI, cumulus-oocyte complexes are denuded of cumulus cells by gentle pipetting in medium containing hyaluronidase (200 IU mL) (Altermatt et al., 2009); therefore, the oocyte morphology can be critically evaluated. In depth evaluation allows for the possibility of correlating parameters of oocyte morphology with embryo quality, viability, and pregnancy outcome. Many morphological parameters can be assessed such as oocyte shape, cytoplasm color and granulation, regularity and thickness of the zona pellucida, size of the perivitelline space, presence of vacuoles, presence or absence of germinative vesicles and the polar body (Navarro et al., 2009, Xia, 1997). Increased incidence of morphological abnormalities of oocytes with increasing age has been reported in several animal species, including mice, rats and rabbits (Werner, 1991).

2.4.2 Cumulus-Oocyte Complex

The oocyte is surrounded by the zona pellucida, corona radiata and the cumulus cells, which facilitates maturation and development into a mature oocyte capable of fertilization. The combined oocyte, zona pellucida, corona radiata and cumulus cells create the cumulus-oocyte complex (COC) (Dandekar, 1992). The close association between the oocyte and cumulus cells allows one cell type to influence the other.

2.4.3 Zona Pellucida

The zona pellucida contributes to the fertilization process, providing species-specific sites for sperm binding, inducing the sperm acrosome reaction, serving as a block to polyspermy, and providing initial protection to the early embryo. The zona pellucida in the equine is composed of four glycoproteins of differing molecular masses. The equine glycoproteins share common antigens with bovine and porcine glycoproteins (Liu and Shivers, 1982). The origin of glycoproteins varies depending on the species. In the mouse, glycoproteins are exclusively synthesized by the oocyte (Flechon et al., 1984), whereas in the cat, the granulosa cells are responsible for synthesis (Jewgenow and Rudolph, 2001). In other species such as the pig, cow, dog, rabbit, monkey, human and horse, the oocyte and follicle cells are responsible for glycoprotein synthesis (Dunbar et al., 1994). The ability to synthesize zona pellucida glycoproteins is strongly correlated with the developmental capacity of the cumulus-oocyte complex (Kölle et al., 1998).

Each glycoprotein has a specific role in the fertilization process, as well as collectively contributing to zona pellucida structure. Glycoprotein C provides the receptors for binding of sperm and is involved in the induction of the sperm acrosome

reaction (Wassarman et al., 2004). After the penetration of the first sperm, zona pellucida A and C glycoproteins modify their chemical structure so that the zona pellucida serves as a site of block to polyspermy (Wassarman et al., 2005, Sun et al., 2005). As the oocyte is fertilized and the early embryo is transported through the oviduct, the zona pellucida is thought to provide initial protection and support (Sun et al., 2005).

The glycoprotein matrix layer of the zona pellucida is 12 to 15 μm thick in cattle (Lindner and Wright, 1983), about 7 μm thick in rodents, and 13 to 15 μm thick in humans (De Vos and Van Steirteghem, 2000). The mean thickness of the zona pellucida is greater for in vivo matured and ovulated oocytes than in vitro matured oocytes in humans (Funahashi et al., 2000). Zona pellucida thickness was significantly thinner in old mares (≥20 yr) versus young mares (4 to 9 yr) and was not correlated with early developmental competence of embryos produced by ICSI (Altermatt et al., 2009). Oocytes from the preovulatory follicles of old mares had significantly larger inner zona pellucida space volumes and perivitelline space volumes than oocytes from young mares. Larger inner zona pellucida space volume and perivitelline space volume were associated with decreased cleavage and pregnancy rates after ICSI (Altermatt et al., 2009).

Zona pellucida morphology in human oocytes has been evaluated more thoroughly than in horses. The zona pellucida has been critically evaluated for the effect of thickness and thickness variation on pregnancy evaluation. Oocytes harvested from three age groups of women (younger than 30 yr, 30 to 34 yr and older than 35) were used. Zona pellucida thickness declined with age, and the zona pellucida thickness from patients older than 35 yr was significantly lower than women in the younger age groups.

Variation in the zona pellucida thickness was significantly higher for embryos that resulted in pregnancy versus those that did not. Pregnancy rates of 50%, 17%, and 0% were noted for zona pellucida thickness variation of greater than 20%, less than 20% and less than 15%, respectively (Sun et al., 2005, Gabrielsen et al., 2000). In human embryos, thinner versus thicker zona pellucidae indicates an increased potential for implantation and pregnancy development. Human embryos with morphology scores less than 1.5 (indicating higher quality) had relatively thin zona pellucidae and higher zona pellucida thickness variation values are compared with embryos graded higher than 1.5 (Gabrielson et al., 2000, Høst et al., 2002).

Transmission electron microscopic comparison of oocyte morphology in young (3 to 10 yr) versus old (> 19 yr) mares revealed minor differences in the cytoplasm. The area of ooplasm occupied by cortical granules tended to be lower in old mares versus young mares, and the area of oocyte occupied by smooth endoplasmic reticulum surrounded by mitochondria tended to be higher (Carnevale et al., 1999). Significantly more oocytes from old mares contained large vesicles (vesicles occupying >1% the total area of the oocyte) than young mares when evaluated under light microscopy (Carnevale et al., 1999). In the human, oocytes with dark cytoplasm versus without dark cytoplasm and numerous vacuoles or fragments in the perivitelline space led to poor quality embryos and lower pregnancy rates (5.5 % versus 29.4%) (Loutradis et al., 1999). Woman older than 35 yr had a higher proportion of oocytes with cytoplasmic inclusions (glycogen granules, lipid droplets and fibrous structures), which were highly correlated with lower fertilization rates and poor embryo quality (Xia, 1997). Oocytes with

darkened and granular centers often fail to fertilize and have reduced developmental potential (Bedford and Kim, 1993). Oocytes with vacuoles or smooth endoplasmic reticulum clusters in the cytoplasm can have normal fertilization with ICSI, but blastocyst formation and pregnancy rates are reduced (Serhal et al., 1997, Otsuki et al., 2004). In the bovine, dark ooplasm indicates an accumulation of lipids and good developmental potential; light ooplasm indicates a low density of organelles and poor developmental potential; black ooplasm indicates aging and low developmental capacity (Wang and Sun, 2007). In the pig, dark granulated ooplasm is a normal occurrence and indicates good developmental capacity; in contrast, dark ooplasm in the human indicates low oocyte quality. The bovine compact cumulus-oocyte complex has a translucent ooplasm, although the expanded oocyte complex has a dark ooplasm (Wang and Sun, 2007). Most oocytes with expanded cumulus in cattle exhibit poorer developmental potential than with compact cumulus; however, the reverse seems to be true for the equine (Hinrichs and Williams, 1997). In the rat, maternal age is associated with an increased proportion of oocytes exhibiting undulations in the nuclear membrane. The nucleoplasm appears denser and the nuclear membrane thicker. The number of cortical granules is decreased, the microvilli are shorter and their frequency along the plasma membrane is reduced (Tarin,1996).

2.4.4 First Polar Body

Polar body morphology in human oocytes has remained a controversial indicator of oocyte viability. Fertilization, cleavage rates, and embryo quality resulting from ICSI of oocytes with an enlarged first polar body were significantly lower than for oocytes with

an intact first polar body of normal size or with a fragmented first polar body (Navarro et al., 2009). Other researchers found similar results (Ebner et al., 1999, 2000 and Xia, 1997); however, in a conflicting study, polar body morphology was not predictive of fertilization rate, cleavage rate, or embryo quality (Ciotti et al., 2004). Variation in the predictive value of polar body morphology could be a result of exogenous ovarian stimulation. Oocytes retrieved after ovarian stimulation showed different grades of first polar body morphology because of varying stages of nuclear maturation (Ciotti et al., 2004). There were no significant differences between oocytes with an intact first polar body of normal size and oocytes with a fragmented first polar body. Normally developed polar bodies, as well as normal position and morphology of the two pronuclei, including the development of the nucleolar precursor bodies, are associated with good quality embryos and favorable pregnancies (Younis et al., 2008). Fertilization rate and embryo quality were significantly better from oocytes with intact polar bodies, normal perivitelline space and without cytoplasmic inclusions (Xia, 1997, Suppinyopong et al., 2000). Aged rats have decreased oocyte viability associated with a reduced potential to form a polar body and the tendency of oocytes to degenerate when placed in culture (Peluso, 1980).

2.5 The Embryo

2.5.1 Morphology

Morphological assessment of uterine equine embryos is important to assess viability, predict pregnancy rates and maximize use of recipients (McKinnon and Squires, 1988). In cattle, sheep and rabbit embryos, results from the use of dye-exclusion tests,

live-dead stains, enzyme activity values and glucose uptakes correlate well with embryo morphologic features and survival after transfer (Lindner and Wright, 1983); however, less research in this area has been conducted on equine embryos (McKinnon and Squires, 1988). Morphological evaluation has remained the most commonly performed technique due to the practicality of field use in commercial operations.

Morphological criteria that have been used when evaluating embryos include rate of cell division, number of blastomeres, size, shape, symmetry, cytoplasmic appearance and presence of anucleate cytoplasmic fragments (Høst et al., 2002), color, compactness of blastomeres, size of perivitelline space, number of extruded and degenerate cells, and developmental stage compared to embryo age (McKinnon and Squires, 1988). Fertilization is recognized by second polar body formation or by cleavage (McKinnon and Squires, 1988). First cleavage is not completed for approximately 24 h, with each subsequent cleavage requiring approximately 12 h (McKinnon and Squires, 1988). The ability of fertilized oocytes to cleave at an early stage (typically 25-27 h) after sperm injection reflects an intrinsic developmental potential (Shoukir et al., 1997). For early embryo (day 2 to 3) analysis, blastomere fragmentation is frequent and is classified based on percentage of embryo volume occupied by fragmentation (Borini et al., 2005); implantation rates decline with an increasing percentage of fragmentation (Alikani et al., 1999). Blastomeres of uneven size could be the result of uneven distribution of genetic material, with higher percentage of chromosomal aberrations and reduced implantation potential (Hardarson et al., 2001).

Embryo grading varies between labs, taking into account subjectivity and personal preference. The primary basis for most classification systems is the assignment of subjective grades to embryos between 1 and 5. For cattle, grade 1 (excellent) embryos are spherical, with uniform cells, color and texture. Grade 2 (good) embryos may have a few extruded blastomeres, irregular shapes or trophoblast separation. Grade 3 (fair) embryos have definite problems, extruded blastomeres, degenerate or collapsed blastomeres. Grade 4 (poor) embryos have severe problems, collapsed blastocoele, numerous extruded blastomeres, and degenerate cells with a viable inner cell mass. Grade 5 (dead) includes dead embryos or unfertilized oocytes (Lindner and Wright ,1983).

Morphological observations of equine embryos, such as extruded blastomeres, darkened cells and collapsed blastocoele, are good indicators of altered embryo viability and predictive of success of embryo transfer (Carnevale et al., 2000). Pregnancy rates were significantly lower for embryos with quality scores ≥ 3 at early pregnancy. Pregnancy rates are lower after transfer of small (100 to 299 μm) embryos or morulae if they are delayed in development (Carnevale et al., 2000). A delay in embryo development could be caused by intrinsic defects in embryos, exposure to poor environment in the oviduct and/or uterus or postovulatory insemination (Ginther, 1992).

Human embryos have been assessed for viability by different methods. Measurements of embryos must be noninvasive and rapid; routinely, embryos are selected on the basis of morphology and rate of development (Sakkas et al., 2001). The most widely used criteria are cell number and morphology (Cummins et al., 1986). Embryos are graded as top quality if they have 4 to 5 blastomeres on day 2 and at least 7

blastomeres on day 3 after fertilization, absence of multinucleated blastomeres and <20 percent fragmentation on day 2 and day 3 after fertilization (Gardner and Sakkas, 2003). Early cleaving embryos have significantly higher implantation and pregnancy rates (Gardner and Sakkas, 2003). Ideal features in the pronucleate embryo that may indicate high viability include: number and distribution of nuclear precursor bodies in each pronucleus of fertilized zygotes, orientation of pronuclei relative to the polar bodies, alignment of pronuclei and nucleoli, appearance of the cytoplasm, nuclear membrane breakdown and early cleavage to the two-cell stage (Hawley et al., 1995, Gardner and Sakkas, 2003). Blastocysts can be scored according to expansion state of the blastocoelic cavity and the number and cohesiveness of the inner cell mass and trophectodermal cells (Sakkas et al., 2001). Zygotes with equality between the nuclei had 49.5% blastocyst formation and those with unequal sizes, numbers or distribution of nucleoli had 28% blastocyst formation (Scott et al., 2000). Thorough evaluation and critical grading of embryos allows for better selection of quality embryos and maximization of embryo use.

Morphological grading of bovine embryos is similar to equine embryos. The diameter of a bovine embryo is estimated to be 150 to 190 μm, which is unchanged from the zygote diameter until blastocyst expansion (Lindner and Wright, 1983). The most prevalent developmental stages obtained after uterine flushes were morulae and compact morulae on day 5 to 6, early blastocysts and blastocysts on day 7, and blastocyst, expanded blastocysts and hatched blastocysts on days 8 and 9 post onset of estrus (Lindner and Wright, 1983). For in vivo matured embryos, the stage of development was

not predictive of pregnancy rate, whereas embryo quality was a more accurate predictor of pregnancy rate (Lindner and Wright, 1983).

To better evaluate and study the kinetics of embryo development, 320 in vitro matured and fertilized bovine oocytes were evaluated by time-lapse cinematography (Somfai et al., 2009). Embryos were classified according to their cleavage pattern at the first cell division. From the embryos examined, 285 embryos cleaved; 119 had two blastomeres of the same size (normal cleavage – NC), 49 had two blastomeres with multiple small fragments (multiple fragment – MF), 34 had two blastomeres and a protrusion (protrusion – PT), 45 showed direct cleavage from 1 cell to 3 or 4 blastomeres (3-4 BL), and 60 oocytes cleaved to two blastomeres of different sizes (unequal blastomeres – UB). The first and second cell cycles of viable embryos (that could develop to the blastocyst stage) were significantly shorter than those of nonviable embryos (24.9 ±0.3h and 8.7 ±0.1h versus 26.6 ±0.7h and 10.0 ±0.1h, respectively). The duration of the 1-cell stage in the normal cleavage embryos was significantly shorter than that of the multiple fragment, protrusion, direct cleavage and unequal blastomeres groups (24.7 ±0.4h, 26.6 ±0.5h, 26.3 ±0.6h, 26.0 ±0.2h, 27.7 ±0.9h, respectively). The percentage of normal cleavage embryos was similar to the direct cleavage embryos (66.9% and 56.7%, respectively), but significantly higher than multiple fragment, protrusion and unequal blastomeres groups (40.5%, 26.5% and 35.6%, respectively). Most of the direct cleavage embryos showed abnormal ploidy. Results of first cell cycle, and cleavage pattern during first cell division, can be a marker of developmental

competence and should be considered for the selection of good quality embryos for embryo transfer (Somfai et al., 2009).

2.6 The Effect of Maternal Aging on Oocytes

2.6.1 Chromosomal Abnormalities/DNA damage

Oocyte abnormalities related to maternal age include meiotic incompetence or inability to resume or complete meiotic maturation. Such abnormalities could result in oocytes incapable of fertilization or in errors that do not prevent fertilization but cause genetic abnormalities that compromise embryo viability and can lead to spontaneous abortion or fetal abnormalities (Armstrong, 2000). Cytoplasmic deficiencies related to maternal age are less understood, but contribute to cell cycle regulation including regulation of meiosis and can contribute to failure of meiotic maturation (Armstrong, 2000).

DNA and oxidative damage has been associated with many types of cellular damage, causing failure of tissues and organs of different species. DNA fragmentation associated with apoptotic death could be one of the causes for poor oocyte quality and lower fertility in aged mice (Fujino et al., 1996). The free radical theory of aging emphasizes peroxidative damage to nuclear DNA or cell membranes as the main mechanism for the decay of cellular function with age (Harman, 1956). This theory has been modified to include oxygen radical damage to mitochondria (Shigenaga et al., 1994). Mitochondrial DNA is the major target for oxidative attack because of the location near the inner mitochondrial membrane where oxidants are formed, as well as the lack of protective histones and DNA repair activity. Oxidative stress may be generated by

oocytes and by the surrounding cells and cause a decrease in oocyte viability with a subsequent reduction in pregnancy rates and an increase in spontaneous abortion rates (Meldrum, 1993). Oocytes from older women are 3.3 times more likely to contain a common 5 kb mitochondrial deletion than oocytes from younger women (Keefe et al., 1995).

Potential effects of age-associated oxidative stress on oocytes and embryos include cytoskeletal alterations, increased frequency of aneuploidy, inhibition of extrusion of the first polar body and an increased incidence of cellular fragmentation and gross abnormal morphology (Tarin, 1996). Age-associated oxidative stress of oocytes may inhibit fertilization by counteracting the reduction potential of oocytes. Specifically, damage to cytoskeletal fibers could cause deterioration of the machinery involved in cortical granule exocytosis. Damage may induce spontaneous release of cortical granules with resulting changes in zona pellucida glycoproteins (sperm penetration would be prevented) or inhibit or delay cortical granule release after entry of the fertilizing sperm into the oocyte (polyspermy would be promoted) (Tarin, 1996). Among these cytoskeletal alterations, the increased frequency of aneuploidy, inhibition of extrusion of the first polar body and increased incidence of cellular fragmentation and gross abnormal morphology are of particular concern, especially for those perimenopausal women planning pregnancies (Tarin, 1996).

Oxidative stress can induce chromosomal damage and aneuploidy. Aneuploidy is a concern for human patients. In the human, 10-30% of fertilized oocytes have the wrong number of chromosomes (Koehler et al., 1996). Aneuploidy can be associated with

fertilization failure, failure of implantation and pregnancy losses. The incidence of aneuploidy increases with age, either resulting in monosomy or trisomy, most of which are maternal in origin (Hassold et al., 1996, Armstrong, 2000). The observed level of aneuploidy in humans varies: in newborns 0.3% (trisomy 21 and sex-chromosome trisomies being most prevalent); in stillbirths 4% (similar abnormalities to newborns) and in clinically recognized abortions (between 6 to 8 weeks and 20 weeks gestation) 35% of such conceptions are trisomic or monosomic for various chromosomes (Hassold et al., 1996), whereas in early missed abortion, the rate of chromosomally abnormal embryos is higher (67-75%) (Philipp et al., 2003). Trisomy can contribute to fetal losses and has adverse effects in offspring ranging from physical, behavioral or intellectual impairment (Hassold et al., 1996). Reduced oxygen supply to the developing follicle may lead to premature oocyte maturation, early oocyte atresia and abnormal polar body morphology. Reduced ovarian blood flow and the resulting hypoxia has been linked to defects in the oocyte spindle, which could cause aneuploidy and abnormal polar body morphology, as well as impinge on ATP content and impair oocyte developmental capacity (Younis et al., 2008). In horses, the rate of aneuploidy has been estimated to be twice as prevalent as hyperhaploidy, or around 33% (Brinsko et al., 1995).

The degree of apoptosis increases with advancing age. Oocytes from human donors were evaluated in culture for maturation and signs of apoptosis (shrinkage, cytoplasmic condensation, membrane blebbing, and/or fragmentation of oocytes into apoptotic bodies of unequal sizes) (Høst et al., 2002). Maturation rates of oocytes were highest in women 21 to 30 yr ($p < 0.005$). Signs of apoptosis increased as oocytes were

recovered from older women (17.1%, 37.7% and 52.3% from women 21 to 30, 31 to 40 and 41 to 50 yr, respectively). There were no significant differences in time of in vitro maturation for oocytes collected from the different age groups. Women who conceived had fewer granulosa cells that were apoptotic than women who did not conceive. No correlation was found between the degree of apoptosis in the cumulus cells to the zona pellucida thickness and zona pellucida thickness variation. A significantly lower number of apoptotic cumulus cells were associated with oocytes that were fertilized compared to unfertilized oocytes (Høst et al., 2002).

CHAPTER 3.0

Experiment I and II

3.1 Introduction

Oocyte morphology and quality has been implicated as a cause of reduced fertility in many mammalian species (Carnevale et al., 1995, Krisher, 2004). However, morphology and quality are difficult to assess. Many subjective measures have been developed, although few definitive measures have been found. In vitro fertilization techniques are unsuccessful in horses (Palmer et al., 1991, Hinrichs et al., 2002, Kölle et al., 2007); therefore alternative assisted reproductive technologies have been researched. The use of ICSI has increased for horses and allows for morphologic evaluations of oocytes and early embryos prior to transfer into recipients, which can be correlated to future pregnancy development. ICSI allows utilization of poor quality or limited amounts of semen (Lazarri et al., 2002). ICSI circumvents the need for sperm to bind and penetrate the zona pellucida, one of the major blocks to in vitro fertilization (Hinrichs et al., 2002). Selected oocyte morphological parameters have been correlated with pregnancy in humans and horses (Sun et al., 2005, Gabrielsen et al., 2000, Host et al., 2002, Altermatt et al., 2009). Early embryo development is routinely assessed in human in vitro fertilization clinics, and aspects have been associated with further development

(Gardner and Sakkas, 2003, Hawley et al., 1995). Objective measurements of morphology to assess oocyte and early embryo morphology could be used to critically evaluate and identify embryos with the highest developmental potential, to optimize recipient utilization and pregnancy rates.

The hypothesis of the first study was that oocyte morphology differs with donor mare age and corresponds with developmental potential after ICSI. Objectives for the first study were to compare: 1) oocyte donor age with oocyte morphology and developmental competency after ICSI, and 2) oocyte morphology with developmental competency (cleavage, early pregnancy, late pregnancy and pregnancy loss) after ICSI.

The hypothesis of the second study was that early embryo morphology differs with donor mare age and corresponds with developmental potential after ICSI. Objectives for the second study were to compare developmental potential of ICSI-produced embryos with: 1) oocyte donor age, and 2) cleavage characteristics, and 3) rate of embryonic development.

3.2 Materials and Methods

Data were obtained for 93 mares in a clinical ICSI program during the 2007 and 2008 breeding seasons. Oocytes were collected from the mares from March to September of 2007 and from February to August of 2008 at latitude 40° N (Fort Collins, CO, USA).

Mares weighed between 385 and 590 kg and were nonlactating (n=88) and lactating (n=5) and of a variety of light-horse breeds. Mares were of known ages between 3 and 28 yr. Some mares were admitted to the program with histories of subfertility

(failure to become pregnant or produce embryos), and some mares were admitted to the program with histories of normal fertility or no reproductive history. To compare age effects, mares were grouped into four age groups, 3 to 13, 14 to 19, 20 to 23, and 24 to 27 yr. In general, mares ≥14 yr were consistent in presentation to the program, with previous reproductive success (pregnancies or embryo donation) but with recent histories of fertility problems, such as chronic uterine infections. Younger mares, 3-13 yr, presented with variable histories and were subsequently categorized into mares with known fertility potential (known history of fertility or normal young mare without a recent history of performance or medical procedures) or unknown fertility potential (unknown reproductive history and recently or currently performing or with major medical issues). Many of the mares in performance competition potentially were treated with performance enhancing drugs that could interfere with fertility (Maher et al., 1983, Squires et al., 1985)

During the breeding seasons, the mares were housed under natural and artificial lighting in stalls with runs. Mares were fed diets specifically formulated for their needs and primarily consisting of grass and/or alfalfa hay and a complete feed (Safe Choice, Nutrena Horse Feed, Cargill Inc.). The mares were provided controlled, mild exercise as limited by age and physical capabilities.

Reproductive tracts were examined using a transrectal ultrasound machine with a 5 MHz linear transducer (Aloka SSD, 500V; Aloka Science and Humanity, Wallingford, CT, USA). Follicle and oocyte maturation were induced when a follicle approximately 35 mm in diameter, endometrial edema and relaxed cervical tone were observed, although

criteria were altered for individual mares or cycles as required. The following agents were used for maturation induction: 1) human chorionic gonadotopin (hCG;1500-2500 IU, IV, Intervet, Millsboro, DE, USA) with administration of deslorelin (0.75-1.5 mg, IM, Francks Pharmacy, Ocala, FL, USA) approximately 4 h later (n=344); 2) recombinant LH (rLH; 0.75 mg, IV, AspenBio Pharma Inc., Castle Rock, CO, USA) (n=14) or rLH with deslorelin (n=3); 3) deslorelin (n=7) or 4) a compounded combination of deslorelin and hCG (2000IU hCG and 1.5mg deslorelin, IV, American Pharmacy Solutions, Mobile, AL, USA) (n=28). For some cyles (n=17), signs of impending ovulation were observed with ultrasound, and maturation induction agents were not administered prior to oocyte collection.

Horses and procedures for Experiment 2 were a subgroup from Experiment 1. For this experiment, retrospective analyses were done on early-stage embryos. However, additional criteria were used to minimize variability because of the smaller number of embryos. Therefore, donors 3 to 13 yr were not categorized; only early embryos transferred surgically were included and embryos for which a high quality image was not available were excluded.

3.2.1 Oocyte Collection and Culture

Most oocytes were collected the day after administration of a follicle maturation agent. Prior to aspirations, mares were premedicated with flunixin meglumine (1.1 mg/kg intravenously, Prevail, VetOne, MWI, Meridian, ID). Each mare was restrained in stocks and sedated (xylazine HCl, 0.4-0.8 mg/kg, IV, TranquiVed, Vedco, St. Joseph, MO and butorphanol tartrate, Torbugesic, 0.02- 0.05 mg/kg, IV, Fort Dodge, MWI, Meridan, ID).

Rectal tone and contractions were reduced by administration of *N*-butylscopolammonium bromide (Buscopan, 0.3mg/kg intravenous, Boehringer-Ingelheim, St. Joseph, MO). Oocyte collections were performed using a linear transducer contained in a plastic casing with a needle guide. The transducer was introduced into the anterior vagina lateral to the cervix. A double lumen, 12-gauge needle (Cook Veterinary Products, Australia or SurgiVet, Waukesha, WI) was inserted into the needle guide, and the needle was attached to a pump (UltraQuiet, COOK, Australia) set at 150 mmHg. Follicles were simultaneously aspirated and flushed with approximately 150 mL of warmed (38.5 C) medium (EmCare Complete Flush Solution, ICP-Bio, Auckland, New Zealand) supplemented with 10 IU/mL of heparin (Calbiochem; La Jolla, CA or Sigma, St. Louis, MO).

Oocytes were identified and transferred into 2.5 mL of culture medium [TCM-199 with Earles' salts (Bio Whittaker, Walkersville, MD) with 0.2 mM pyruvate, and 25 μg/ml gentamycin] and cultured at 38.5° C in 6% CO_2 in air.

3.2.2 Intracytoplasmic sperm injection

Prior to ICSI, the cumulus oocyte complexes were denuded by pipetting in a buffered commercial medium (G-MOPS; Vitrolife AB, Goteborg, Sweden) with 0.5% bovine serum albumin and containing hyaluronidase (200 IU/ml, Sigma, St. Louis, MO). Sperm were obtained from fresh, cooled or frozen semen from stallions (n=75) of variable fertility. Approximately 100 μl of extended fresh or cooled semen was placed in 1 ml of F-CDM [CDM (Olson and Seidel, 2000) supplement with 0.5% fatty acid free BSA, 2 mM caffeine and 2 μg/ml heparin] in a round bottom tube. A straw of frozen

semen selected for use with ICSI was cut into approximately a 5 mm section under liquid nitrogen. The cut section was placed into 1 ml of F-CDM at 38.5° C, 6% CO_2 and air, in a round bottom tube. The tube was held at a 45° angle for 20 minutes before 500 µl of supernatant was placed into 2 ml of pre-equilibrated F-CDM and centrifuged at 300-320g for 5 min. The supernatant was removed, and 1 µl of the pellet was placed in 5µl of G-MOPS containing 0.5% BSA and 5% polyvinylpyrrolidone (ICN Biomedicals Inc., Aurora, Ohio, USA). Sperm were selected based on progressive motility and normal morphology and injected into oocytes using a micromanipulator (Narishige Group, Japan) and Piezo-driven injection system (Prime Tech Inc., Japan).

3.2.3 Oocyte morphologic parameters

After removal of cumulus cells, images of oocytes were captured using an inverted microscope (Nikon Instruments, Inc., USA) at 200X; images were captured at the widest subjective measurement and at the approximate plane of the first polar body. Oocytes were measured using digital calipers within a computer software program (Spot Software, Diagnostic Instruments, Inc. Sterling Heights, MI, USA). Oocyte diameter (OD) was obtained as the average of two measurements at the widest and narrowest oocyte axes. Oocyte volume (OV) was calculated using a modified oblate spheroid formula $4/3\pi a^2$ b, where a and b were the radii from the widest and narrowest axes, respectively. Zona pellucida thickness without matrix (ZPT) was determined as the average of two measurements of the zona pellucida thickness at 180° intervals. Zona pellucida thickness with matrix (ZPTM) was the average of four measurements of the zona pellucida thickness, including the surrounding matrix, at 90° intervals. Inner zona

pellucida diameter (IZPD) was determined as the average of two measurements from the inner border of the zona pellucida and 90° apart. Inner zona pellucida volume (IZPV) was calculated using modified oblate spheroid formula, $4/3\pi a^2$ b, with a and b from the radii of the IZPD. Ooplasm diameter (OpD) was defined as the average of two measurements from the outer edge of the ooplasm and at 90° angles. Ooplasm volume (OpV) was calculated using the formula $4/3\pi r^3$ based on average OD. Pervitelline space volume (PVSV) was calculated as the difference between IZPV and OpV (Altermatt et al., 2009a).

3.2.4 Embryo morphologic parameters

The ICSI-produced embryos were evaluated for the following binomial characteristics: 1) even or uneven blastomeres, ratio of blastomere sizes ≥ 60:40, 2) rapid cleavage, three or four blastomeres imaged within 24 h after ICSI, 3) large fragments of membrane-bound ooplasm of inadequate size for blastomeres, and 4) extrusion of the oolemma from the zona pellucida. Small, numerous fragments of ooplasm associated with the early embryo were estimated as a percentage of the total blastomere volume. Blastomere numbers were recorded at observations and at transfer.

3.2.5 Embryo culture

After ICSI (Day 0), potential zygotes were placed in embryo culture medium [DMEM/F12 (Sigma, St. Louis, MO, USA) containing 10% fetal calf serum] at 38.5 C in 5% CO_2, 5% O_2 and 90% N_2. The injected oocytes were observed during Day 1 to 3 to assess cleavage and embryo development. Cleavage was defined as presence of two or more symmetrical blastomeres within the zona pellucida. Zygotes were graded by one of

the two technicians, and images were captured using an inverted microscope (Nikon Instruments, Inc., USA) at 200X.

3.2.6 Embryo transfers

Recipient mares were approximated as between 3 and 15 years, as based on dental wear characteristics. Prior to use, potential recipients' reproductive tracts were evaluated by ultrasound and palpation for normal luteal appearance, uterine and cervical tone, and absence of pathology. Embryos resulting from oocytes were transferred surgically (n=222) or nonsurgically (n=11) into recipients' oviducts or uteri, respectively. However, retrospective morphology assessments for early embryos were only done for early embryos that were transferred surgically into oviducts and for which appropriate images were obtained (n=178). Surgical transfers were done through standing flank laparotomies (Carnevale et al 1993). Preference was given to transfers on the contralateral side to ovulation to avoid the developing corpus luteum. Prior to transfer, mares were premedicated with flunixin meglumine (1.1 mg/kg, IV, Prevail, VetOne, MWI, Meridian, ID) , restrained in stocks and sedated (xylazine HCl, 0.4-0.8 mg/kg IV, acepromazine maleate 0.04-0.1 mg/kg IV and butorphanol tartrate, 0.02-0.05mg, IV). Mares were clipped and aseptically prepared from the last rib to the tuber coxae. A line block was performed using approximately 100 mL of 2% lidocaine (Vedco, St Joseph, MO, USA). An incision, approximately 12-cm in length, was made halfway between the last rib and the tuber coxae at the level of the base of the tuber coxae. The ovary was gently retracted from the abdomen to allow a sterilized, glass pipette, containing the embryo, to be placed into the infundibulum of the oviduct. The embryo was placed approximately 3 cm into

the oviduct. The abdomen was closed in a four layer closure and recipients were housed in 12' x 12' runs for 2 weeks for recovery. Post operatively, mares were administered antibiotics (sulfamethoxazole and trimethoprim, 6.8 mg/kg, per os, q 12 h; Amneal Pharmaceuticals, Glasgow, KY, USA) for 7 days and phenylbutazone (2 g, per os q 24 hours, 2.2 mg/kg, Vedco, St Joseph, MO, USA) for 3 days. Recipient mares received exogenous progesterone or a progestin to assure pregnancy maintenance.

For nonsurgical transfers, embryos were transferred between 6 to 9 days after ICSI into the uteri of recipients synchronized from -4 to +2 days from the day of ICSI (day 0). One noncyclic recipient was synchronized by exogenous steroids prior to use. For nonsurgical transfer, a transfer gun (IMV Technologies, L'Aigle, Normandy, France L'Aigle, Normandy, France) was used to place the embryo into the body of the uterus. Embryos that had poor development, excessive fragmentation, or degenerate cells were not transferred.

3.2.7 Pregnancy Detection

Beginning at 11 days after ICSI, transrectal ultrasound was used to examine uteri of recipients' for the presence of an embryonic vesicle; recipients in which no vesicles were detected were examined until 17 days after ICSI. Diameters of embryonic vesicles were measured using internal ultrasound calipers. Embryonic vesicles detected by day 17 after ICSI were classified as early embryonic vesicles (EEV). The uteri of pregnant recipients were periodically examined for the detection of an embryo proper with heartbeat and continued normal development. Embryonic vesicles detected by day 17 after ICSI were classified as early pregnancy detection (EPD), and pregnancies diagnosed

at later stages of development (approximately 50 days) were classified as late pregnancy detection (LPD). Pregnancy loss (PL) was defined as the loss of the pregnancy between early and late examinations.

3.4 Statistics

Oocyte data among age groups were analyzed using SAS/STAT software, Version 9.2 for Windows, copyright SAS Institute Inc., Cary, NC, USA. The GLIMMIX procedure was used for most analyses, because it could analyze both normal and binary responses. Missing values for time of administration of maturation-inducing agents were estimated based upon the means for time of administration of compounds working directly at the level of the follicle (hCG or rLH) or pituitary (deslorelin). The mean time of the two types of maturation agents was used as a covariate for statistical analyses. Data for volume measurements were log base 10 transformed to equalize variances for large and small means and to normalize the distribution of residuals. Tukey-Kramer was used to test for differences among groups. Students' t-tests were used to compare morphology measurements for oocytes that did or did not cleave, form blastocysts and develop into pregnancies. Repeated oocyte collection values for mares within the same year were evaluated as the same mare. Since there were only a few mares repeated in both years, they were considered as different mares in the two years.

For embryo morphology parameters, age groups were analyzed using the GLIMMIX procedure to determine overall differences, and least squares means ±SEM. Binomial data were compared using GLIMMIX or Fisher's exact test if cell numbers were low

CHAPTER 4.0

Results

Experiment I: Oocyte Morphology

Images from 401 oocytes were used for morphological measurements. Cleavage rates were similar for oocytes from all mares, regardless of age or category (Known or Unknown) (Figure 1). The highest ($p<0.05$) early pregnancy rates were observed in mares 3 to 13 yr Known and 14 to 19 yr. When compared to mares 14 to 19 yr, early pregnancy rates tended ($p=0.06$) to be lower for mares 20 to 23 yr and were lower ($p=0.02$) for mares 24 to 27 yr. However, oocytes from mares 3 to 13 yr Unknown had limited developmental capacity, with the numerically lowest early pregnancy rate (15%), which was similar ($p>0.1$) to mares ≥20 yr. Pregnancy losses had a linear increase with age, with a significant increase from donors 3 to 13 yr Known to donors ≥20 yr; pregnancy losses tended ($p=0.07$) to be higher for mares 24 to 27 yr versus 14 to 19 yr (Figure 1).

Oocyte volume (OV) and inner zona pellucida volume (IZPV) were not different ($p>0.05$) among groups (Figure 2). Ooplasm volume (OpV) was larger ($p<0.05$) for oocytes from mares 14 to 19 and 20 to 23 yr than for mares 3 to 13 yr Known or mares 24 to 27 yr; ooplasm volume was similar for mares 3 to 13 yr Unknown and all other groups (Figure 2). Volume of the perivitelline space (PVSV) was similar ($p=0.2$) between

mares 3 to 13 yr Unknown and mares 20 to 23 yr, but smaller ($p \leq 0.05$) for mares 3 to 13 yr Unknown than the other age groups (Figure 2).

Oocyte diameter (OD) differed ($p=0.05$) between oocytes from donors 3 to 13 yr Known and 14 to 19 yr, but oocyte diameter was similar among all other groups (Figure 3). Inner zona pellucida diameter (IZPD) differed ($p=0.03$) only between mares 14 to 19 yr and mares 3 to 13 yr Unknown, with oocytes from mares 14 to 19 yr having the largest numerical IZPD and mares in the Unknown group having the smallest IZPD (Figure 3). Ooplasm diameter (OpD) was smaller ($p \leq 0.02$) for oocytes from mares 3 to 13 yr Known than from mares 14 to 19 or 20 to 23 yr. Ooplasm diameter was numerically smallest for mares 24 to 27 yr, but similar for mares in the 3 to 13 yr categories (Figure 3).

Diameter of the zona pellucida (ZPT) was not different ($p>0.05$) among groups. The diameter of the zona pellucida with the surrounding matrix (ZPTM) was greater ($p<0.05$) for mares 3 to 13 yr Unknown than for the other groups (Figure 4).

Experiment II: Early Embryo Morphology

Records and images were used for 178 ICSI-produced embryos from 86 donor mares and 59 stallions. Mean donor age tended ($P \leq 0.1$) to be lower for early embryos that did, versus did not result in early embryonic vesicles detected within recipients' uteri (14.97 ±0.68 and 16.67 ±0.75 yr, respectively), and the mean age was lower ($p<0.05$) for donors whose embryos did, versus did not result in late embryonic vesicles (13.75 ±0.80 and 17.0 ±0.63 yr). Binomial morphology characteristics of early embryos were not

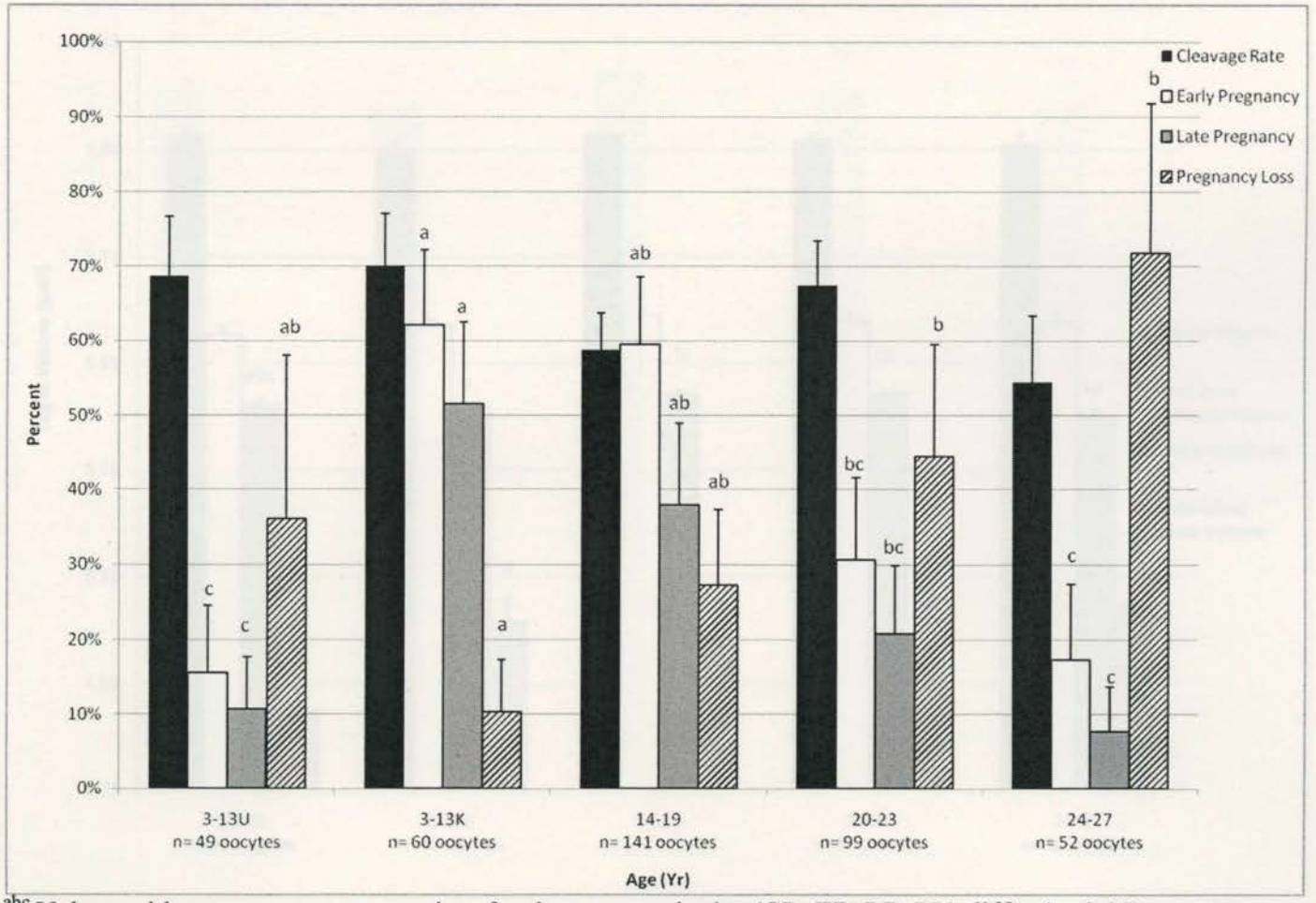
associated with mare age or with development to early or late embryonic vesicles (Table 5).

When embryos from donors 3 to 13, 14 to 19, 20 to 23 and 24 to 27 yr were compared, mean number of hours from ICSI to embryo cleavage and to embryo transfer did not differ among ages (Table 6). However, the number of blastomeres per embryo at transfer was higher ($P<0.05$) for mares 20 to 23 yr than younger mares (Table 6). In contrast, the time to transfer per cell (hours/cell) tended ($P\leq0.1$) to be different among groups and differed ($p<0.05$) among mares 3 to 13 and 20 to 23 yr (Table 6). The estimated percentage of small fragmentation was not different among groups. Diameter of the embryonic vesicle at first observation and the growth rate of embryonic vesicles (mm/day) in recipients' uteri did not differ by age (Table 6). However, day of first detection of embryonic vesicles tended ($P\leq0.1$) to differ among groups, with vesicles detected earlier ($p<0.05$) for donors 14 to 19 than 20 to 23 yr (Table 6).

The mean hours from ICSI to first cleavage tended ($P\leq0.1$) to be longer for embryos that did not produce early embryonic vesicles, but did not differ for detection of late pregnancies (Table 7). Hours to transfer and number of blastomeres per embryo at transfer did not differ for early and late pregnancy detection (Table 7). However, the rate of embryo development (hours per cell) prior to transfer was faster ($P<0.05$) for embryos that did, versus did not produce an early pregnancy and tended ($P\leq0.1$) to be faster for embryos that did, versus did not produce a late pregnancy (Table 7). Embryonic vesicles, first detected earlier (12 versus 13 days, respectively) within recipients uteri, resulted in

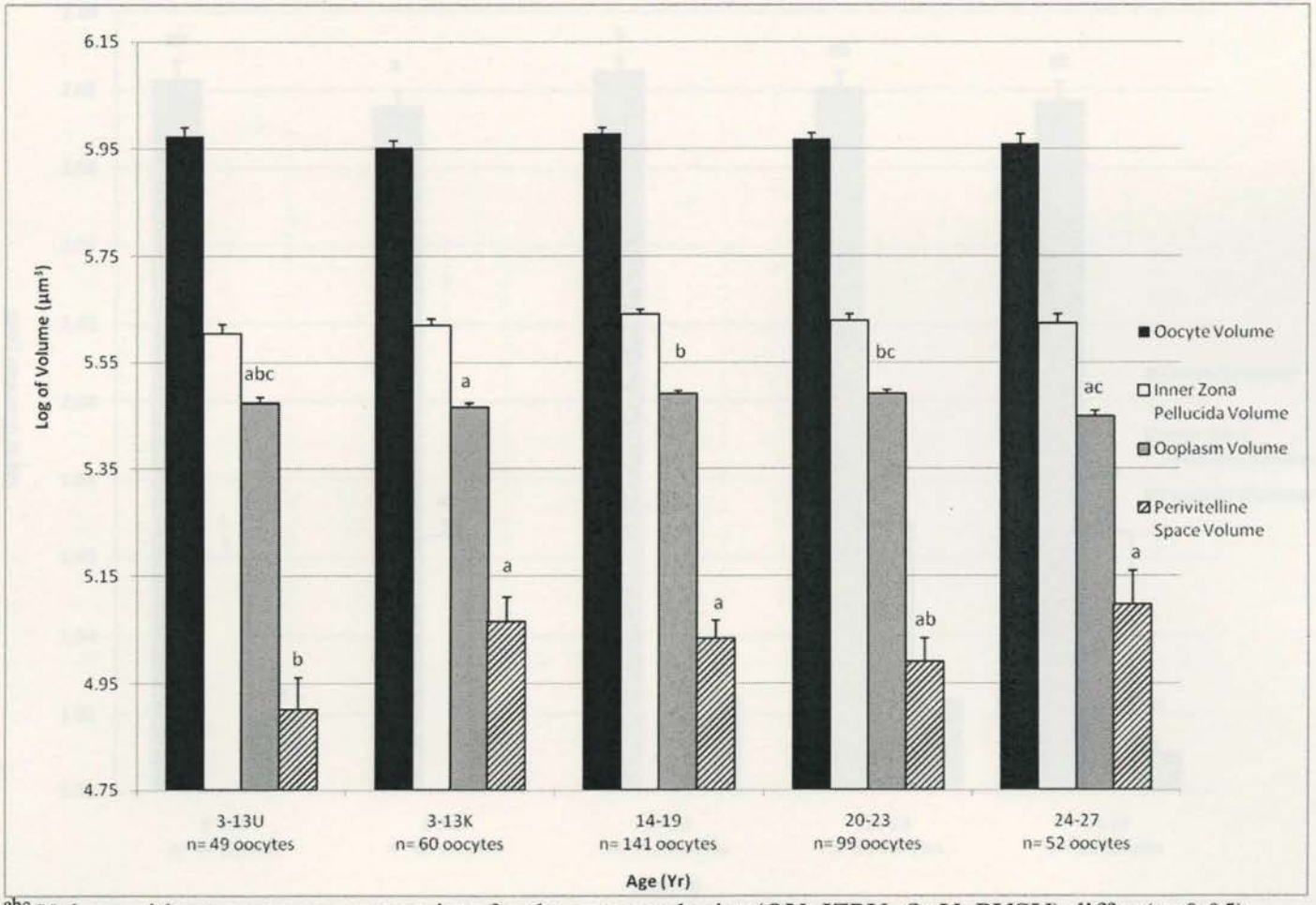
more ($P<0.05$) late pregnancies (Table 7). Embryonic vesicles that increased in diameter faster were more often ($p<0.05$) maintained to the late pregnancy stage (Table 7).

Figure 1: Number of cleaved oocytes, number of early pregnancies (11-16 d), and number of late pregnancies (50 d), pregnancy loss (number of late pregnancies per early pregnancy) for oocytes from donors 3-13 Unknown, 3-13 Known, 14-19, 20-23 and 24-27 yr.



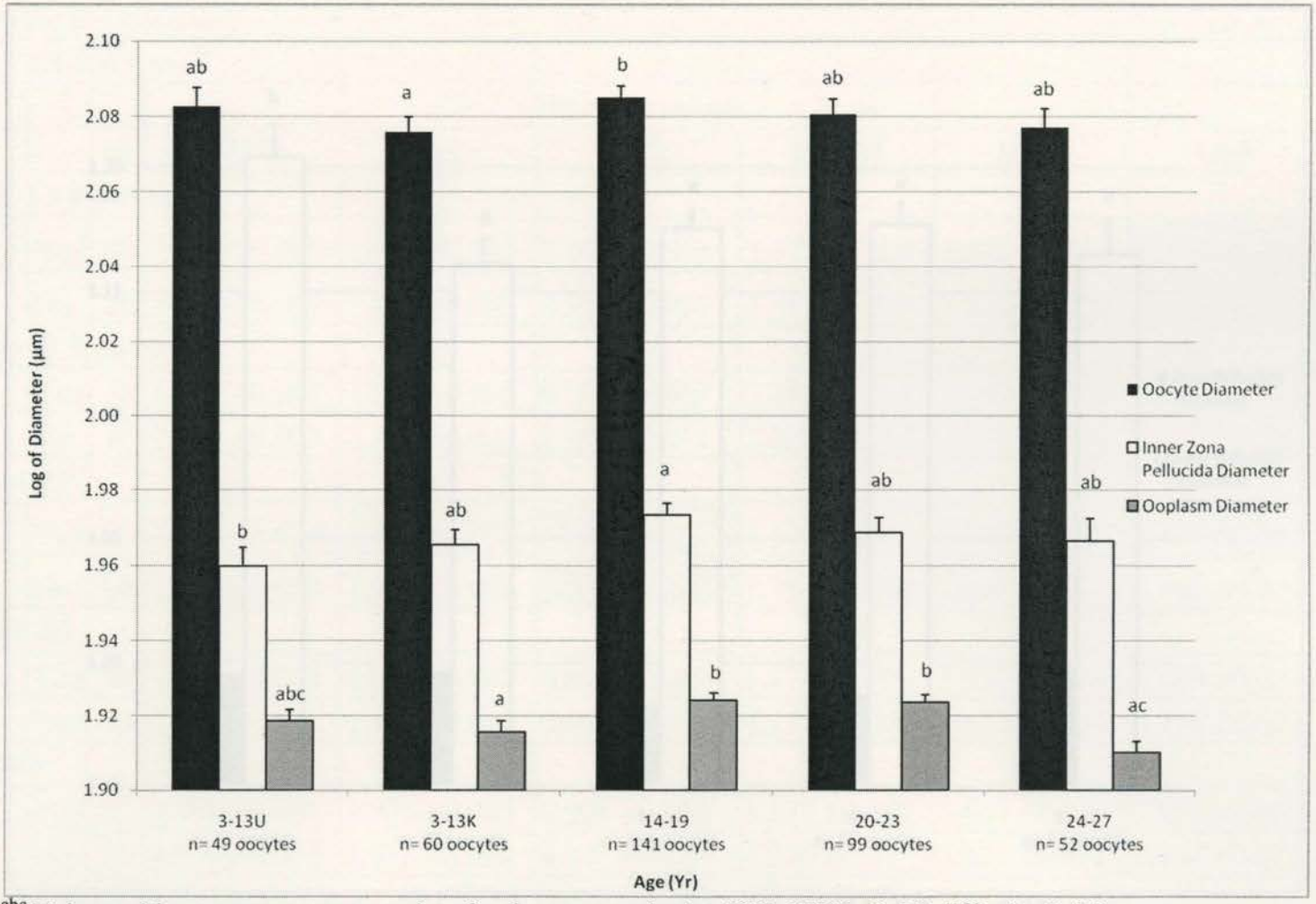
[abc] Values without common superscripts for the same endpoint (CL, EP, LP, PL) differ ($p<0.05$)

Figure 2: Mean ±SEM of log transformed data (Base 10) for oocyte volume (OV), inner zona pellucida volume (IZPV), ooplasm volume (OpV) and perivitelline space volume (PVSV) for oocytes from donors 3-13 Unknown, 3-13 Known, 14-19, 20-23 and 24-27 yr.



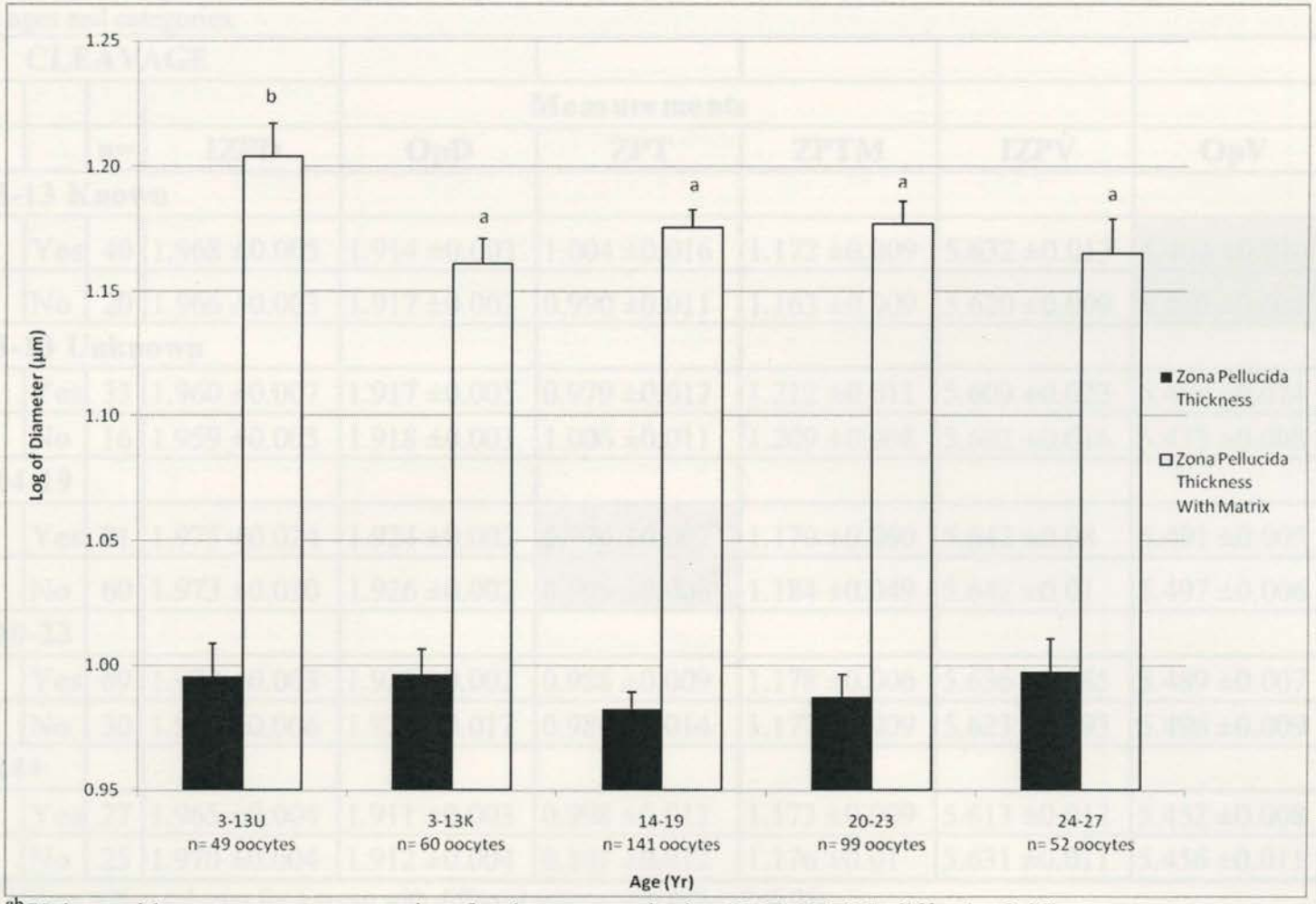
[abc] Values without common superscripts for the same endpoint (OV, IZPV, OpV, PVSV) differ ($p<0.05$)

Figure 3: Mean ±SEM of log transformed data (Base 10) of oocyte diameter (OD), inner zona pellucida diameter (IZPD), ooplasm diameter (OpD) for oocytes from donors 3-13 Unknown, 3-13 Known, 14-19, 20-23 and 24-27 yr.



[abc] Values without common superscripts for the same endpoint (OD, IZPD, OpD) differ ($p<0.05$)

Figure 4: Mean ±SEM of log transformed data (Base 10) of zona pellucida thickness (ZPT) and zona pellucida thickness with matrix (ZPTM) for oocytes from donors 3-13 Unknown, 3-13 Known, 14-19, 20-23 and 24-27 yr.



[ab] Values without common superscripts for the same endpoint (ZPT, ZPTM) differ ($p<0.05$).

Table 1: Mean ±SEM for log transformed (base 10) morphological measurements for inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), inner zona pellucida volume (IZPV) and ooplasm volume (OpV) for injected oocytes that cleaved (yes) versus did not cleave (no) within mares of different ages and categories.

CLEAVAGE								
				Measurements				
		n=	IZPD	OpD	ZPT	ZPTM	IZPV	OpV
3-13 Known								
	Yes	40	1.968 ±0.005	1.914 ±0.003	1.004 ±0.016	1.172 ±0.009	5.632 ±0.013	5.460 ±0.010[c]
	No	20	1.966 ±0.003	1.917 ±0.003	0.990 ±0.011	1.163 ±0.009	5.620 ±0.009	5.470 ±0.008[d]
3-13 Unknown								
	Yes	33	1.960 ±0.007	1.917 ±0.005	0.979 ±0.017	1.212 ±0.011	5.609 ±0.023	5.469 ±0.014
	No	16	1.959 ±0.005	1.918 ±0.003	1.006 ±0.011	1.209 ±0.008	5.602 ±0.016	5.473 ±0.008
14-19								
	Yes	81	1.975 ±0.024	1.924 ±0.002	0.974 ±0.007[a]	1.170 ±0.060	5.642 ±0.08	5.491 ±0.005
	No	60	1.973 ±0.030	1.926 ±0.002	0.995 ±0.008[b]	1.184 ±0.049	5.642 ±0.01	5.497 ±0.006
20-23								
	Yes	69	1.972 ±0.003	1.923 ±0.002	0.988 ±0.009	1.178 ±0.006	5.636 ±0.085	5.489 ±0.007
	No	30	1.966 ±0.006	1.926 ±0.017	0.986 ±0.014	1.177 ±0.009	5.623 ±0.093	5.496 ±0.009
24+								
	Yes	27	1.965 ±0.004	1.911 ±0.003	0.998 ±0.012	1.173 ±0.009	5.613 ±0.012	5.452 ±0.008
	No	25	1.970 ±0.004	1.912 ±0.004	0.101 ±0.012	1.176 ±0.01	5.631 ±0.011	5.456 ±0.011

[a,b] Values within columns for a group with different superscripts differ ($P \leq 0.05$)

[c,d] Values within columns for a group with different superscripts tended to differ ($P \leq 0.1$)

Table 2: Mean ±SEM for log transformed (base 10) morphological measurements for inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), inner zona pellucida volume (IZPV) and ooplasm volume (OpV) for injected oocytes that developed into early pregnancy (11-16 d) (yes) versus did not develop (no) within mares of different ages and categories.

EARLY PREGNANCY								
				Measurements				
		n=	IZPD	OpD	ZPT	ZPTM	IZPV	OpV
3-13 Known								
	Yes	24	1.961 ±0.004[a]	1.914 ±0.003[a]	1.002 ±0.014	1.160 ±0.010	5.609 ±0.011[c]	5.461 ±0.010[a]
	No	12	1.975 ±0.006[b]	1.927 ±0.004[b]	0.970 ±0.018	1.162 ±0.014	5.647 ±0.016[d]	5.500 ±0.011[b]
3-13 Unknown								
	Yes	7	1.966 ±0.010	1.910 ±0.005	0.986 ±0.027	1.223 ±0.014	5.619 ±0.032	5.450 ±0.016
	No	19	1.960 ±0.007	1.918 ±0.004	1.011 ±0.013	1.200 ±0.012	5.606 ±0.024	5.471 ±0.011
14-19								
	Yes	42	1.976 ±0.004	1.925 ±0.002	0.973 ±0.009	1.170 ±0.009	5.646 ±0.013	5.495 ±0.006
	No	28	1.971 ±0.004	1.922 ±0.003	0.989 ±0.012	1.183 ±0.010	5.637 ±0.012	5.484 ±0.009
20-23								
	Yes	28	1.972 ±0.006	1.926 ±0.004	1.009 ±0.013[c]	1.187 ±0.01	5.637 ±0.019	5.500 ±0.011
	No	33	1.971 ±0.004	1.920 ±0.003	0.975 ±0.012[d]	1.175 ±0.008	5.633 ±0.013	5.479 ±0.008
24+								
	Yes	6	1.963 ±0.009	1.918 ±0.002	0.968 ±0.021[c]	1.133 ±0.024[a]	5.607 ±0.025	5.474 ±0.006
	No	17	1.970 ±0.005	1.910 ±0.004	1.005 ±0.009[d]	1.185 ±0.01[b]	5.630 ±0.016	5.448 ±0.011

[a,b] Values within columns for a group with different superscripts differ ($P \leq 0.05$)

[c,d] Values within columns for a group with different superscripts tended to differ ($P \leq 0.1$)

Table 3: Mean ±SEM for log transformed (base 10) morphological measurements for inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), inner zona pellucida volume (IZPV) and ooplasm volume (OpV) for injected oocytes that developed into late pregnancies (50 d) (yes) versus did not develop into late pregnancies (no) within mares of different ages and categories.

LATE PREGNANCY								
				Measurements				
		n=	**IZPD**	**OpD**	**ZPT**	**ZPTM**	**IZPV**	**OpV**
3-13 Known								
	Yes	21	1.962 ±0.004	1.914 ±0.004[a]	1.010 ±0.015[a]	1.162 ±0.011	5.612 ±0.013	5.460 ±0.012[a]
	No	15	1.972 ±0.005	1.925 ±0.003[b]	0.965 ±0.015[b]	1.158 ±0.011	5.637 ±0.014	5.494 ±0.010[b]
3-13 Unknown								
	Yes	4	1.970 ±0.011	1.920 ±0.004	1.014 ±0.024	1.237 ±0.020	5.614 ±0.038	5.480 ±0.012
	No	22	1.960 ±0.007	1.915 ±0.004	1.001 ±0.014	1.201 ±0.010	5.605 ±0.022	5.462 ±0.011
14-19								
	Yes	30	1.977 ±0.005	1.926 ±0.003	0.972 ±0.011	1.163 ±0.011	5.646 ±0.016	5.496 ±0.008
	No	40	1.972 ±0.003	1.923 ±0.002	0.985 ±0.010	1.185 ±0.008	5.639 ±0.011	5.487 ±0.007
20-23								
	Yes	19	1.966 ±0.005	1.922 ±0.004	1.007 ±0.017	1.186 ±0.014	5.617 ±0.016	5.485 ±0.013
	No	42	1.973 ±0.005	1.923 ±0.003	0.983 ±0.011	1.178 ±0.007	5.642 ±0.014	5.489 ±0.008
24+								
	Yes	2	1.970 ±0.019	1.923 ±0.004	0.952 ±0.049	1.096 ±0.075[a]	5.633 ±0.056	5.488 ±0.012
	No	21	1.968 ±0.004	1.911 ±0.003	0.999 ±0.009	1.178 ±0.009[b]	5.624 ±0.014	5.451 ±0.009

[a,b] Values within columns for a group with different superscripts differ ($P \leq 0.05$)

[c,d] Values within columns for a group with different superscripts tended to differ ($P \leq 0.1$)

Table 4: Mean ±SEM for log transformed (base 10) morphological measurements for inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), inner zona pellucida volume (IZPV) and ooplasm volume (OpV) for injected oocytes that were maintained to late pregnancy (50 d) after early pregnancy detection (16 d) (no) versus not maintained to late pregnancy (yes) within mares of different ages and categories.

PREGNANCY LOSS								
				Measurements				
		n=	**IZPD**	**OpD**	**ZPT**	**ZPTM**	**IZPV**	**OpV**
3-13 Known								
	Yes	3	1.960 ±0.006	1.916 ±0.005	0.944 ±0.029	1.141 ±0.005	5.599 ±0.019	5.468 ±0.016
	No	21	1.962 ±0.004	1.914 ±0.004	1.010 ±0.015	1.162 ±0.011	5.611 ±0.013	5.460 ±0.012
3-13 Unknown								
	Yes	3	1.960 ±0.021	1.897 ±0.003[a]	0.948 ±0.052	1.204 ±0.018	5.603 ±0.062	5.409 ±0.010[a]
	No	4	1.970 ±0.011	1.920 ±0.004[b]	1.014 ±0.024	1.237 ±0.020	5.632 ±0.038	5.480 ±0.012[b]
14-19								
	Yes	12	1.973 ±0.006	1.925 ±0.004	0.977 ±0.016	1.190 ±0.013	5.645 ±0.021	5.493 ±0.011
	No	30	1.977 ±0.005	1.926 ±0.003	0.972 ±0.011	1.163 ±0.011	5.646 ±0.016	5.496 ±0.043
20-23								
	Yes	9	1.983 ±0.015	1.935 ±0.007	1.013 ±0.020	1.188 ±0.012	5.677 ±0.049	5.525 ±0.021
	No	19	1.966 ±0.005	1.922 ±0.004	1.007 ±0.017	1.186 ±0.014	5.617 ±0.016	5.485 ±0.013
24+								
	Yes	4	1.959 ±0.019	1.916 ±0.001[c]	0.976 ±0.026	1.152 ±0.014	5.595 ±0.030	5.455 ±0.004[c]
	No	2	1.970 ±0.019	1.923 ±0.004[d]	0.952 ±0.049	1.096 ±0.075	5.633 ±0.056	5.488 ±0.012[d]

[a,b] Values within columns for a group with different superscripts differ ($P \leq 0.05$)

[c,d] Values within columns for a group with different superscripts tended to differ ($P \leq 0.1$)

Table 5: Mare age (mean ±SEM) and number of early pregnancies (≤17 d) and late pregnancies (50 d) per transferred early embryos associated with various embryo morphology parameters.

Endpoint	Age (yr)	Early Pregnancy (%)	Late Pregnancy (%)
Blastomere Equality			
Even	15.27 ±0.83	34/67 (51)	23/67 (34)
Uneven	16.10 ±0.66	54/105 (51)	40/105 (38)
Rapid Cleavage			
≥ 3 cells by 24 h post ICSI	16.50 ±1.25	10/20 (50)	8/20 (40)
≤ 2 cells by 24 h post ICSI	15.72 ±0.55	80/158 (51)	57/158 (36)
Large Fragment			
Present	16.86 ±1.00	16/36 (44)	11/36 (31)
Not Present	15.54 ±0.58	74/142 (52)	54/142 (38)
Extruded Membrane			
Present	13.67 ±2.50	3/6 (50)	3/6 (50)
Not Present	15.88 ±0.52	87/172 (51)	62/172 (36)

No significant differences were noted within columns for the same endpoint

Table 6: Cleavage and developmental characteristics (means ±SEM) of ICSI-produced equine embryos from mares 3-13, 14-19, 20-23 and 24-27 yr.

	Mare Age (yr)			
Endpoint	**3 to 13**	**14 to 19**	**20 to 23**	**24-27**
ICSI to cleavage (hr)	24.22 ±0.42	23.83 ±0.38	24.47 ±0.50	24.22 ±0.80
ICSI to transfer (hr)	32.72 ±1.45	30.98 ±1.38	35.33 ±1.80	34.37 ±2.92
Cells at transfer	2.58^{a} ±0.18	2.73^{a} ±0.17	3.60^{b} ±0.22	3.13^{ab} ±0.34
Time to transfer per cell (h/cell)	13.08^{a} ±0.42	12.12^{ab} ±0.40	11.42^{b} ±0.52	11.71^{ab} ±0.84
Fragmentation (%) *	18.39 ±1.47	18.56 ±1.41	16.76 ±1.82	19.96 ±2.80
Day of detection of embryonic vesicle	12.09^{ab} ±0.31	11.84^{a} ±0.30	13.00^{b} ±0.37	12.50^{ab} ±0.82
Diameter (mm) of embyonic vesicle at detection	5.41 ±0.59	5.77 ±0.58	6.17 ±0.71	7.82 ±1.54
Growth rate of embryonic vesicle (mm/d)	0.44 ±0.04	0.48 ±0.04	0.48 ±0.05	0.63 ±0.11

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

[c,d] Values within rows with different superscripts tended to differ ($P \leq 0.1$)

* Fragmentation: subjective percentage of fragmented ooplasm per total blastocyst

Table 7: Mean ±SEM hours from ICSI to observation of cleavage and transfer, number of cells at transfer, developmental rate (hours per cell) at transfer, amount of fragmented ooplasm per total blastocyst volume, day, size and growth rate (mm per day) of embryonic vesicle upon initial detection in recips' uteri as associated with early and late pregnancy detection.

	Early Pregnancy		Late Pregnancy	
Endpoint	**Positive**	**Negative**	**Positive**	**Negative**
Time from ICSI to cleavage (hr)	23.75^{c} ±0.21	24.53^{d} ±0.40	23.82 ±0.30	24.29 ±0.31
Time from ICSI to transfer (hr)	31.74 ±1.06	33.59 ±1.15	32.45 ±1.25	32.80 ±1.00
Number of cells at transfer	2.96 ±0.15	2.87 ±0.15	3.02 ±0.17	2.86 ±0.13
Time to transfer per cell (h/cell)	11.63^{a} ±0.26	12.81^{b} ±0.35	11.74^{c} ±0.26	12.51^{d} ±0.32
Fragmentation (%)	17.63 ±1.11	19.19 ±1.10	16.85 ±1.29	19.31 ±0.97
Day of detection of embryonic vesicle	12.24 ±0.18	N/A	11.90^{a} ±0.19	13.12^{b} ±0.36
Diameter (mm) of embyonic vesicle at detection	5.90 ±0.32	N/A	6.22 ±0.41	5.08 ±0.47
Growth rate of embryonic vesicle (mm/d)	0.48 ±0.02	N/A	0.51^{a} ±0.03	0.38^{b} ±0.03

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

[c,d] Values within rows with different superscripts tended to differ ($P \leq 0.1$)

* Fragmentation: percentage of fragmented ooplasm per total blastocyst volume

CHAPTER 5.0

Discussion and Conclusions

5.1 Discussion

ICSI is utilized to assist fertilization and facilitate production of valuable foals from subfertile mares and stallions. In preparation for ICSI, the oocyte is stripped of surrounding cumulus cells, and the oocyte can be observed for morphologic evaluation and assessment. This assessment provides information as to the potential viability of the oocyte and age-associated changes in oocyte morphology. With utilization of the Piezo drill, high fertilization and cleavage rates (>80 % cleavage) have been obtained (Choi et al., 2002, Galli et al., 2002).

Oocyte morphology assessments in the present study involved 93 mares and 75 stallions. Mares and stallions were of variable ages, fertility, performance and health status. Semen used in the present study was fresh, cooled, frozen or frozen after epididymal collections. No difference was noted in cleavage or embryo development in previous research among oocytes injected with sperm from stallions with good, poor or no fertility when motile sperm were selected for ICSI (Lazarri et al., 2002, Choi et al., 2002, Serhal et al., 1997). Stallion age has no effect on live foal rates (McDowell et al., 1992). Due to the large number of stallions, unknown stallion ages and different types of semen, stallion effects were not analyzed in the present study.

Mares younger than 13 yr were typically admitted to the clinical program as oocyte donors for stallions with fertility problems or limited semen availability. Mares older than 13 yr are often admitted to the program with breeding histories and past evidence of normal oocyte quality; but recent histories as unsuccessful embryo donors due to chronic endometritis or other reproductive problems. With advancing age in mares, uterine functional and histological changes are identified (Carnevale and Ginther, 1992), which can lead to an inadequate uterine environment for embryo development and subsequent loss. Mares older than 15 yr have more extensive intrauterine fluid accumulations, more inflammatory cell infiltrations and more fibrotic changes on uterine biopsy than mares 5 to 7 yr (Carnevale and Ginther, 1992). In the present study, a few mares, older than 13 yr, had severe debilitating diseases or medical conditions and were excluded from analysis.

The youngest age group (3 to 13yr) was further classified into Known and Unknown mares, based on incoming history. Mares with known fertility history, such as live foal(s) or previous successful embryo or oocyte donors, were classified as "Known". Mares with unknown fertility history, including mares recently racing or performing, mares recovering from surgery (orthopedic and soft tissue), mares with medical conditions with continuing treatment, mares with recent granulosa cell tumor removal and mares in poor body condition, were classified as "Unknown" due to unknown effects of exercise, exogenous compounds, and stress on fertility. Intense exercise has been shown to affect mares' cycles by increasing the interovulatory length, delaying follicle deviation and increasing growth rate of the second largest follicle (Kelley et al., 2009). Exercise was associated with a decrease in the number of embryos recovered per cycle

and the quality score of embryos (Mortensen et al., 2009). Studies to evaluate the effect of administration of anabolic steroids on fertility found that estrous behavior in all treatment groups was suppressed and the number of ovulations was decreased (Squires et al., 1985, Maher et al., 1983). Compounds such as anabolic steroids lower fertility and cause an increased rate of early embryonic death (Squires et al., 1985, Maher et al., 1983). The effects of long term medical treatments on reproductive performance have not been studied; therefore all mares on long term medical treatments were classified as unknown.

Increasing maternal age correlates with decreasing fertility, including decreased uterine function, decreased rates of embryonic detection, higher incidences of embryonic loss and reduced oocyte quality and morphology in both humans and horses (Carnevale and Ginther, 1992, Vanderwall et al., 1989, Carnevale, 2008, Carnevale et al., 1995, Werner et al., 1991, Krisher, 2004).

In the present study, mares 3-13 yr Unknown had significantly lower early and late pregnancy rates than the other ages, as well as a much higher pregnancy loss rate than the 3 to 13 yr Known mares. This may be due to the intense performance and competition that the Unknown group had been involved in. Intense exercise has been shown to impact fertility (Mortensen et al., 2009). Many horses in competition are administered exogenous compounds for performance; some of these agents have been associated with a decrease in fertility which could explain the lower pregnancy rates, as well as the increase pregnancy loss (Maher et al., 1983, Squires et al., 1985).

Mares for the study were divided into age groups based on demonstrated fertility decline (McDowell et al., 1992); groups included young, teenage, early twenties and aged

mares. Fertility has been shown to begin to decline in the early teenage years (McDowell et al., 1992); therefore, mares less than 13 yr were considered young. Older mares produce fewer foals, which is most likely due to higher levels of embryonic loss rather than failure to conceive (McDowell et al., 1992, Vanderwall 2008). Fertilization rates in vivo were not different for young, normal or aged, subfertile mares (Ball et al., 1986, Ball et al., 1989) or young and old pony mares (Carnevale et al., 1993). In the present study, cleavage rates for injected oocytes were similar for all age groups and classifications. ICSI minimizes confounding variables that could occur with natural fertilization, suggesting that the reduced pregnancy rates and increased pregnancy loss rates may be due to an inherent defect in the oocyte. Results of previous oocyte transfer studies have demonstrated that defective oocytes account for most of the subfertility in old mares independent of the effects of oviductal and uterine pathology (Carnevale et al., 1995).

Fertility and pregnancy rates have been shown to decline with advancing age from around 60% in younger mares (5 to 7 yr) to 30% in older mares (≥15 yr) (Vanderwall et al., 1989, Ginther, 1992). In the present study, early and late pregnancy rates were highest and pregnancy loss rates were lowest for mares 3 to 13 yr Known and 14 to 19 yr, which is in agreement with what has been historically shown.

Fertility decreases in the teenage years (McDowell et al., 1992). Embryonic loss rates of 20-30% have been detected in mares greater than 18 yr of age (Vanderwall, 2008). Early embryonic loss is a result of failure in development due to poor quality oocytes or exposure to inadequate environment (Ball et al., 1986). Aged mares have a higher incidence of early embryonic loss between the days 15 and 50 versus young mares (39% versus 17% loss, respectively) (Villahoz et al., 1985). In the present study, early

and late pregnancy rates declined for the early and late twenties. Embryonic loss rates increased in a linear fashion with age of oocyte donor. There were more mares in the present study aged 24-27 yr than most populations assessed for embryonic loss. ICSI involves an increased manipulation of the cycle, oocyte and recipient which could potentially affect embryonic development (Li et al., 2001, Galli et al., 2007, Hinrichs et al., 2007, Choi et al., 2002, Galli et al., 2002).

Age-associated morphological changes have been identified in human oocytes that have been correlated with changes in fertility (Xia, 1997, Gabrielson et al., 2000, Høst et al., 2002, Sun et al., 2005, Bedford and Kim, 1993, Serhal et al., 1997, Otsuki et al., 2004, Ebner et al., 1999 and 2000, Ciotti et al., 2004, Younis et al., 2008, Suppinyopong et al., 2000). However, minimal research has been done in the horse. One study evaluated equine oocyte morphology and the correlation with developmental potential (Altermatt et al., 2009). That research involved two distinct age groups (4 to 9 yr and >20 yr) of non-performing mares in good health without many confounding variables. The study involved the use of frozen sperm from a single ejaculate of a young non-performing stallion of known fertility.

In the human and horse, the oocyte and follicle cells are responsible for glycoprotein synthesis (Dunbar et al., 1994). The ability to synthesize zona pellucida glycoproteins is strongly correlated with the developmental capacity of the cumulus oocyte complex (Kolle et al., 1998). Zona pellucida thickness was significantly smaller in old (>20 yr) than young (4 to 9 yr) mares in a previous study; however, no association to developmental competence after ICSI was observed (Altermatt et al., 2009). In the present study zona pellucida thickness was similar among all groups; however,

differences existed within some age groups. Thinner zona pellucida thickness had a tendency to be associated with higher cleavage rates within mares 14 to 19 yr, as well as higher early pregnancy rates for 24 to 27 yr mares. A thicker zona pellucida tended to be associated with higher early pregnancy rates for 20 to 23 yr mares and was significantly associated with higher late pregnancy rates for 3 to 13 yr mares. The zona pellucida is surrounded by a matrix of amorphous proteinaceous and hyaluronic acid elements associated with the cumulus-oocyte complex and remain attached to the zona pellucida (Dandekar et al., 1992). The zona pellucida is denuded of cumulus cells in the stripping process, which could cause an artifactual alteration in the thickness measurement of the matrix surrounding the oocytes. All of the oocytes undergo stripping with a pipette of similar inner diameter; therefore, any effect of this artifact is theoretically minimal. A thinner zona pellucida thickness with matrix was significantly associated with higher early and late pregnancy rates from mares 24 to 27 yr. In the women, zona pellucida characteristics are associated with womens' age (Sun et al., 2005). Zona pellucida thickness declines with age, women older than 35 yr having the smallest thickness. However, the results of the present study were inconsistent to draw a conclusion regarding zona pellucida morphology and developmental potential. Changes in the zona pellucida could reflect the oocyte and follicle function, functionally could have more of an affect on fertilization in vivo.

Ooplasm volume, inner zona pellucida volume and perivitelline space volume may change as a function of age. Fertilization rate and embryo quality were significantly better from oocytes with normal perivitelline space (Xia, 1997, Suppinyopong et al., 2000). Perivitelline space volume was larger in older women (Xia, 1997). Enlarged

perivitelline space with debris may be related to overmaturity (Xia, 1997). In humans, dark ooplasm and numerous vacuoles or fragments in the perivitelline space lead to poor quality embryos and lower pregnancy rates (Loutradis et al., 1999). Previous research in the horse reported an association between a larger perivitelline space volume and decreased cleavage and pregnancy rates after ICSI (Altermatt et al., 2009). Similar trends were observed in the present study; however, they were not statistically significant.

A smaller inner zona pellucida diameter and volume were associated with higher early pregnancy rates for mares 3 to 13 yr Known. A smaller ooplasm diameter and volume tended to be associated with higher cleavage rates, early pregnancy rates and late pregnancy rates for 3 to 13 yr Known. However, a smaller ooplasm diameter and volume were significantly associated with increased pregnancy loss for 3 to 13 yr Unknown and tended to be associated with increased pregnancy loss for the 24 to 27 yr mares. Previous research did not report any associations between ooplasm volume, ooplasm diameter and cleavage, or blastocyst formation and pregnancy rates (Altermatt et al., 2009).

The mares 3 to 13 yr Unknown contained oocytes that exhibited oocyte morphology that may indicate immaturity. The zona pellucida thickness with matrix and zona pellucida thickness were thicker than all other groups. The inner zona pellucida diameter and volume and the perivitelline space volume were the smallest in this group. Decreased perivitelline space volume may be associated with immaturity; enlarged volumes have been correlated with oocyte over maturity in humans (Xia, 1997), although in depth studies of oocyte maturity in the horse have not been evaluated.

Embryo morphology

The morphology of uterine embryos has been used as a predictive indicator of developmental ability in the mare (Carnevale et al., 2000). However, few early equine embryos have historically been observed and evaluated for morphology and developmental potential.

In the present experiment, morphology was evaluated in ICSI-produced embryos. The presences of blastomeres, rapid cleavage, large fragments or membrane extruded from the zona pellucida were not associated with age and were not predictive of early or late embryo development. Early developmental characteristics have been described for bovine embryos produced by in vitro fertilization using time-lapse photography. Embryos that contained equal blastomeres developed into significantly more blastocysts than embryos displaying multiple fragments, protrusions, and unequal blastomeres (Somfai et al., 2009). Bovine embryos that displayed rapid cleavage showed abnormal ploidy in blastomeres, including haploidy and triploidy. The equine embryos in the present study were not evaluated for ploidy. In horses, the rate of aneuploidy has been estimated to be twice as prevalent as hyperhaploidy, or around 33% (Brinsko et al., 1995). The larger sample size of bovine embryos (285 bovine versus 178 equine embryos) may have revealed morphological differences that were not able to be detected in a smaller sample of equine embryos. Further studies of cleavage patterns in equine embryos produced by ICSI are merited to determine whether or not they exhibit similar developmental markers. On early embryo (day 2 to 3) analysis of in vitro produced human embryos, blastomere fragmentation is frequent and is classified based on percentage of embryo volume occupied by fragmentation (Borini et al., 2005); implantation rates decline with an

increasing percentage of fragmentation (Alikani et al., 1999). In the present study, percentage of fragmentation was similar for all groups, although it washighest for the oldest mares (24 to 27 yr).

In the present experiment, the speed of development appeared to be the most predictive factor of early embryo viability. Fewer hours from ICSI to cleavage and faster development (h/cell to transfer) were associated with more early pregnancies. The time to first cleavage was significantly earlier for embryos that did versus did not result in early pregnancy. Higher pregnancy rates result from human embryos produced by conventional in vitro fertilization and ICSI that show early cleavage (defined as cleavage into two cells by 27 h post injection) (Shoukir et al., 1997, Sakkas et al., 1998). For equine embryos, significantly more early embryonic vesicles were detected from embryos with shorter time to transfer per blastomere, suggestive of faster cell development rates. Late embryonic vesicles also showed this trend, with more 50 d pregnancies from embryos that developed faster. Comparable cell development rates have been observed in ICSI-produced equine embryos, with cleavage to 2 cells observed after 20 h of in vitro culture (Choi et al., 2002), an average of 3-4 cells by 48 h of in vitro culture (Choi et al., 2004a), and an average of >8 cells after 96 hours of in vitro culture (Choi et al., 2002).

Mean age of the oocyte donor tended to be less for donors with early pregnancy detection and was significantly less for donors with late pregnancy detection. Differences in embryonic vesicle detection rates between young and old mares have been observed in previous studies (Ball et al., 1986, 1989, Carnevale et al., 1992, 1993). The day of detection of embryonic vesicles differed among age groups, but did not differ significantly overall. Diameter and growth rate of embryonic vesicles upon detection did

not differ significantly among age groups. Undersized embryonic vesicles are associated with reduced embryo viability and an increased incidence of embryo loss (Ginther et al., 1985), and embryonic vesicles imaged on the first pregnancy exam (Day 12) are less likely to result in embryonic death than vesicles first imaged on later dates (Carnevale et al., 2000). Embryonic vesicle fixation and the detection of an embryo proper occur later in old versus young mares (Carnevale et al., 1992). Late embryonic vesicle detection showed differences in the day of detection and growth rate between mares that did and did not remain pregnant to 50 d. The growth rate of vesicles that remained viable pregnancies was significantly faster than vesicles that were lost (0.5 mm/d versus 0.4 mm/d). The mean diameter of late embryonic vesicles on the day of initial detection was greater (6 mm versus 5 mm) for embryonic vesicles that were versus were not still present at 50 d.

Embryo morphology was not indicative of developmental competence; however, donor age, time to cleavage after ICSI and day of first detection of pregnancy were associated with pregnancy outcome.

5.2 Conclusions

ICSI is a viable method of obtaining pregnancies when more standard in vivo procedures are not an option. Age of the donor affects the potential to produce pregnancies after ICSI. Oocyte morphology measurements were affected by age, but no measurement was consistently predictive of oocyte developmental potential after ICSI. Ooplasm volume showed a significant impact on development, whereas ooplasm diameter, inner zona pellucida diameter and inner zona pellucida volume showed a tendency to affect development.

Donor mare age exerts an effect on rate of cell development, time between injection and transfer, detection of embryonic vesicles, pregnancy development and outcome. Early embryo morphology was not indicative of developmental competence, although faster cleavage and speed of embryo development was associated with a positive pregnancy outcome.

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APPENDIX A

Appendix Table 1:
Mean and log transformed mean (Log base 10) mean ± SEM for oocyte diameter (OD), inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), oocyte volume (OV), inner zona pellucida volume (IZPV), ooplasm volume (OpV), perivitelline space volume (PVSV) and mean percentage ± SEM cleavage (% cleaved per oocyte injected-Cle), early pregnancy detected (% pregnant (≤17d) per oocyte cleaved-EP), late pregnancy (% pregnant (50d) per early pregnancy detected- LP), pregnancy loss (% difference between early and late pregnancy detection- PL) for mares 3-13 yr Unknown

3-13 Unknown (n=49 oocytes)	Mean	Mean (Log) ±SEM
Oocyte Diameter (OD) - μm	121.235	2.083 ±0.005
Inner Zona Pellucida Diameter (IZPD) - μm	91.276	1.959 ±0.005
Ooplasm Diameter (OpD) - μm	82.806	1.918 ±0.003
Zona Pellucida Thickness (ZPT) - μm	10.020	0.996 ±0.013
Zona Pellucida Thickness with Matrix (ZPTM) - μm	16.304	1.210 ±0.013
Oocyte Volume (OV) - μm^3	961670.960	5.978 ±0.016
Inner Zona Pellucida Volume (IZPV)- μm^3	411033.330	5.604 ±0.017
Ooplasm Volume (OpV)- μm^3	298312.860	5.472 ±0.010
Periviteline Space Volume (PVSV) - μm^3	112720.470	4.898 ±0.059
Cleavage (Cle)- (% per oocyte)-%		68.7 ±7.8
Early Pregnancy (EP)- (% per oocyte cleaved) - %		15.5 ±8.6
Late Pregnancy (LP)- (% per oocyte cleaved) - %		10.7 ±6.8
Pregnancy Loss (PL)- (% difference between EP and LP)- %		36.1 ±22.3

Appendix Table 2:
Mean and log transformed mean (Log base 10) mean ± SEM for oocyte diameter (OD), inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), oocyte volume (OV), inner zona pellucida volume (IZPV), ooplasm volume (OpV), perivitelline space volume (PVSV) and mean percentage ± SEM cleavage (% cleaved per oocyte injected-Cle), early pregnancy detected (% pregnant (≤17d) per oocyte cleaved-EP), late pregnancy (% pregnant (50d) per early pregnancy detected- LP), pregnancy loss (% difference between early and late pregnancy detection- PL) for mares 3-13 yr Known

3-13 Known (n=60 oocytes)	Mean	Mean (Log) ±SEM
Oocyte Diameter (OD) - μm	119.675	2.078 ±0.004
Inner Zona Pellucida Diameter (IZPD) - μm	92.758	1.967 ±0.004
Ooplasm Diameter (OpD) - μm	82.442	1.916 ±0.003
Zona Pellucida Thickness (ZPT) - μm	10.008	0.995 ±0.010
Zona Pellucida Thickness with Matrix (ZPTM) - μm	14.748	1.166 ±0.010
Oocyte Volume (OV) - μm^3	919674.160	5.960 ±0.012
Inner Zona Pellucida Volume (IZPV)- μm^3	424768.700	5.620 ±0.013
Ooplasm Volume (OpV)- μm^3	294551.900	5.467 ±0.008
Periviteline Space Volume (PVSV) - μm^3	130216.800	5.082 ±0.046
Cleavage (Cle)- (% per oocyte) -%		70.1 ±6.5
Early Pregnancy (EP)- (% per oocyte cleaved) -%		62.2 ±10.2
Late Pregnancy (LP)- (% per oocyte cleaved) -%		51.6 ±11.3
Pregnancy Loss (PL)- (% difference between EP and LP) -%		10.4 ±6.7

Appendix Table 3:
Mean and log transformed mean (Log base 10) mean ± SEM for oocyte diameter (OD), inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), oocyte volume (OV), inner zona pellucida volume (IZPV), ooplasm volume (OpV), perivitelline space volume (PVSV) and mean percentage ± SEM cleavage (% cleaved per oocyte injected-Cle), early pregnancy detected (% pregnant (≤17d) per oocyte cleaved-EP), late pregnancy (% pregnant (50d) per early pregnancy detected- LP), pregnancy loss (% difference between early and late pregnancy detection- PL) for mares 14-19 yr

14-19 (n=141 oocytes)	Mean	Mean (Log) ±SEM
Oocyte Diameter (OD) - µm	121.922	2.085 ±0.003
Inner Zona Pellucida Diameter (IZPD) - µm	94.340	1.974 ±0.003
Ooplasm Diameter (OpD) - µm	84.159	1.925 ±0.002
Zona Pellucida Thickness (ZPT) - µm	9.713	0.983 ±0.007
Zona Pellucida Thickness with Matrix (ZPTM) - µm	15.118	1.176 ±0.007
Oocyte Volume (OV) - μm^3	972971.600	5.982 ±0.009
Inner Zona Pellucida Volume (IZPV)- μm^3	448637.880	5.642 ±0.009
Ooplasm Volume (OpV)- μm^3	313294.380	5.493 ±0.005
Periviteline Space Volume (PVSV) - μm^3	135343.490	5.041 ±0.033
Cleavage (Cle)- (% per oocyte) -%		58.8 ±4.8
Early Pregnancy (EP)- (% per oocyte cleaved) -%		59.6 ±9.3
Late Pregnancy (LP)- (% per oocyte cleaved) -%		38.0 ±11.1
Pregnancy Loss (PL)- (% difference between EP and LP) -%		27.3 ±10.0

Appendix Table 4:
Mean and log transformed mean (Log base 10) mean ± SEM for oocyte diameter (OD), inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), oocyte volume (OV), inner zona pellucida volume (IZPV), ooplasm volume (OpV), perivitelline space volume (PVSV) and mean percentage ± SEM cleavage (% cleaved per oocyte injected-Cle), early pregnancy detected (% pregnant (≤17d) per oocyte cleaved-EP), late pregnancy (% pregnant (50d) per early pregnancy detected- LP), pregnancy loss (% difference between early and late pregnancy detection- PL) for mares 20-23 yr

20-23 (n=99 oocytes)	**Mean**	**Mean (Log) ±SEM**
Oocyte Diameter (OD) - μm	120.909	2.082 ±0.004
Inner Zona Pellucida Diameter (IZPD) - μm	93.576	1.970 ±0.004
Ooplasm Diameter (OpD) - μm	84.025	1.924 ±0.002
Zona Pellucida Thickness (ZPT) - μm	9.848	0.987 ±0.009
Zona Pellucida Thickness with Matrix (ZPTM) - μm	15.143	1.178 ±0.009
Oocyte Volume (OV) - μm^3	951851.800	5.971 ±0.011
Inner Zona Pellucida Volume (IZPV)- μm^3	437914.160	5.632 ±0.012
Ooplasm Volume (OpV)- μm^3	312241.780	5.491 ±0.007
Periviteline Space Volume (PVSV) - μm^3	125672.380	4.993 ±0.043
Cleavage (Cle)- (% per oocyte) -%		67.5 ±5.8
Early Pregnancy (EP)- (% per oocyte cleaved) -%		30.6 ±10.6
Late Pregnancy (LP)- (% per oocyte cleaved) -%		20.9 ±9.2
Pregnancy Loss (PL)- (% difference between EP and LP) -%		44.5 ±14.7

Appendix Table 5:
Mean and log transformed mean (Log base 10) mean ± SEM for oocyte diameter (OD), inner zona pellucida diameter (IZPD), ooplasm diameter (OpD), zona pellucida thickness (ZPT), zona pellucida thickness with matrix (ZPTM), oocyte volume (OV), inner zona pellucida volume (IZPV), ooplasm volume (OpV), perivitelline space volume (PVSV) and mean percentage ± SEM cleavage (% cleaved per oocyte injected-Cle), early pregnancy detected (% pregnant (≤17d) per oocyte cleaved-EP), late pregnancy (% pregnant (50d) per early pregnancy detected- LP), pregnancy loss (% difference between early and late pregnancy detection- PL) for mares 24-27 yr

24-27 (n=52 oocytes)	Mean	Mean (Log) ±SEM
Oocyte Diameter (OD) - µm	120.173	2.079 ±0.005
Inner Zona Pellucida Diameter (IZPD) - µm	92.846	1.967 ±0.006
Ooplasm Diameter (OpD) - µm	81.644	1.912 ±0.003
Zona Pellucida Thickness (ZPT) - µm	10.009	0.996 ±0.013
Zona Pellucida Thickness with Matrix (ZPTM) - µm	15.038	1.175 ±0.014
Oocyte Volume (OV) - μm^3	935378.390	5.966 ±0.017
Inner Zona Pellucida Volume (IZPV)- μm^3	422616.360	5.622 ±0.018
Ooplasm Volume (OpV)- μm^3	286009.120	5.454 ±0.010
Periviteline Space Volume (PVSV) - μm^3	136607.240	5.088 ±0.063
Cleavage (Cle)- (% per oocyte) -%		54.4 ±8.9
Early Pregnancy (EP)- (% per oocyte cleaved) -%		17.4 ±10.1
Late Pregnancy (LP)- (% per oocyte cleaved) -%		7.7 ±5.8
Pregnancy Loss (PL)- (% difference between EP and LP) -%		71.8 ±20.2

SAMPLE ANOVA TABLE CLEAVAGE FOR OOCYTE DIAMETER FOR KNOWN GROUP

---------- AgeGp=1 K ----------

The TTEST Procedure

Variable: log_OD__Avg

Cle1	N	Mean	Std Dev	Std Err	Minimum	Maximum
0	20	2.0798	0.0142	0.00317	2.0473	2.1106
1	40	2.0767	0.0172	0.00273	2.0394	2.1255
Diff (1-2)		0.00311	0.0163	0.00446		

Cle1	Method	Mean	95% CL Mean		Std Dev	95% CL Std Dev	
0		2.0798	2.0731	2.0864	0.0142	0.0108	0.0207
1		2.0767	2.0712	2.0822	0.0172	0.0141	0.0221
Diff (1-2)	Pooled	0.00311	-0.00583	0.0120	0.0163	0.0138	0.0199
Diff (1-2)	Satterthwaite	0.00311	-0.00532	0.0115			

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	58	0.70	0.4894
Satterthwaite	Unequal	45.361	0.74	0.4615

Equality of Variances

Method	Num DF	Den DF	F Value	Pr > F
Folded F	39	19	1.48	0.3649

SAMPLE ANOVA TABLE EARLY EMBRYONIC VESICLE DETECTION FOR OOCYTE DIAMETER FOR KNOWN GROUP

---------- AgeGp=1 K ----------

The TTEST Procedure

Variable: log_OD__Avg

EEV	N	Mean	Std Dev	Std Err	Minimum	Maximum
0	12	2.0807	0.0162	0.00467	2.0550	2.1255
1	24	2.0734	0.0184	0.00375	2.0394	2.1206
Diff (1-2)		0.00728	0.0177	0.00626		

EEV	Method	Mean	95% CL Mean		Std Dev	95% CL Std Dev	
0		2.0807	2.0704	2.0910	0.0162	0.0115	0.0275
1		2.0734	2.0657	2.0812	0.0184	0.0143	0.0258
Diff (1-2)	Pooled	0.00728	-0.00543	0.0200	0.0177	0.0143	0.0232
Diff (1-2)	Satterthwaite	0.00728	-0.00506	0.0196			

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	34	1.16	0.2523
Satterthwaite	Unequal	24.827	1.22	0.2353

Equality of Variances

Method	Num DF	Den DF	F Value	Pr > F
Folded F	23	11	1.29	0.6785

SAMPLE ANOVA TABLE LATE EMBRYONIC VESICLE DETECTION FOR OOCYTE DIAMETER FOR KNOWN GROUP

---------- AgeGp=1 K ----------

The TTEST Procedure

Variable: log_OD__Avg

LEV	N	Mean	Std Dev	Std Err	Minimum	Maximum
0	15	2.0794	0.0148	0.00381	2.0550	2.1255
1	21	2.0733	0.0196	0.00428	2.0394	2.1206
Diff (1-2)		0.00613	0.0178	0.00601		

LEV	Method	Mean	95% CL Mean		Std Dev	95% CL Std Dev	
0		2.0794	2.0713	2.0876	0.0148	0.0108	0.0233
1		2.0733	2.0644	2.0822	0.0196	0.0150	0.0283
Diff (1-2)	Pooled	0.00613	-0.00608	0.0183	0.0178	0.0144	0.0233
Diff (1-2)	Satterthwaite	0.00613	-0.00551	0.0178			

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	34	1.02	0.3148
Satterthwaite	Unequal	33.873	1.07	0.2922

Equality of Variances

Method	Num DF	Den DF	F Value	Pr > F
Folded F	20	14	1.77	0.2780

APPENDIX B

Introduction

Laparoscopy is routinely being utilized for reproductive applications such as cryptorchid castrations (Hendrickson 2006), granulosa cell tumor removal – laparoscope assisted (Hubert 2006), and ovariectomies (Hendrickson 2006). Experimentally laparoscpy has been used to apply PGE_2 to re-establish oviductal patency (Allen et al 2006) and for tubal ligation (McCue et al 2000). There have been limited studies comparing the effectiveness of laparoscopy and laparotomy for oocyte transfer in the equine. Embryo transfer in transgenic goats comparing laparoscopy and laparotomy transfers showed a significantly better pregnancy rate with the laparoscopic group versus the laparotomy group. Pregnancies rates were 46.1% with the laparoscopic group (n=76 goats) and a 28.6% with the laparotomy group (n=82 goats) (Tae Shin et al 2008). The objectives of this research were to identify the correct portal placement for laparoscopic surgery for oocyte transfer and to identify the ideal pipette or transfer device for use with laparoscopic oocyte transfer.

Materials and Methods:

Non-lactating, light-horse mares, weighing between 385-450 kg and between 4-12 years, were used. Farm records and dental characteristics were used to age the mares; fertility and parity data were not available. Mares were housed under natural lighting in dry lots and fed grass and alfalfa hay. Mares in the study were used as oocyte donors and recipients. Oocytes were collected from June to September 2007 and from June to August 2009 at latitude 40 N (Fort Collins, CO, USA).

Reproductive tracts were examined to monitor status of cycle, endometrial edema and follicle diameter using ultrasonography and a 5 MHz linear transducer (Aloka SSD, 500V; Aloka Science and Humanity, Wallingford, CT, USA). When a follicle, approximately 35 mm in diameter, endometrial edema and relaxed cervical tone were detected, follicle and oocyte maturation were induced with the administration of deslorelin (1.5 mg, IM, Francks Pharmacy, Ocala, FL, USA).

Oocyte Collection

Oocytes were collected the day after administration of a follicle maturation agent. Prior to aspirations, mares were premedicated with flunixin meglumine (1.1 mg/kg IV, Prevail, MWI, Meridian, ID). Mares were restrained in stocks and sedated with xylazine HCl, (100-300 mg, IV) and butorphanol tartrate (5-10 mg, IV). Rectal muscular tone and contractions were decreased by the administration of propantheline bromide (0.05mg/kg, IV; Sigma- Aldrich Corp., St. Louis, MO). Oocyte collections were performed using a linear transducer contained in a plastic casing with a needle guide. The vulva was aseptically prepped, and the transducer was introduced into the anterior vagina lateral to the cervix. A double lumen, 12-gauge needle (Cook Veterinary Products, Australia or SurgiVet, Waukesha, WI) was inserted into the needle guide. The needle was attached to a pump (UltraQuiet, COOK, Australia) set at 150 mmHg. Follicles were simultaneously aspirated and flushed with approximately 150 mL of warmed (38.5°C) medium (EmCare Complete Flush Solution (ICP- Bio, Auckland, New Zealand) supplemented with 10 IU/mL of heparin (Calbiochem; La Jolla, CA or Sigma, St. Louis, MO).

Oocytes were identified using a stereomicroscope and removed from flush medium. Oocyte cumulus complexes were rinsed twice in embryo flush medium and

transferred into 2.5 mL of culture medium (TCM-199, Bio Whittaker, Walkersville, MD) with 0.2 mM pyruvate, and 25 μg/ml gentamycin. Oocytes were cultured at 38.5°C in 6% CO_2 in air.

Oocytes were harvested from donor mares that were then selected to be utilized as recipients for surgery the following day. Mares received between one and four oocytes per transfer, 1 oocytes (n=1), 2 oocytes (n=7), 3 oocytes (n=3) or 4 oocytes (n=1).

Insemination of Recipients

One stallion of known fertility was used in this experiment during both years. The stallion was housed in natural lighting, fed alfalfa and grass hay and housed at the same facility as mares in a 12' x 20' run. Ejaculates were collected using a Colorado model artificial vagina. Semen was collected approximately 12 hours prior to oocyte transfers. The ejaculate was split. One dose (500 X 10^6 PMS) was extended 1:1 with EZ-Mixin CST (ARS, Chino, CA, USA) and inseminated as fresh semen soon after collection; the second dose (1000 x 10^6 PMS) was extended 3:1 with EZ-Mixin CST (ARS, Chino, CA, USA) and cooled to 5° C using an Equitainer (Hamilton Research, Inc., South Hamilton, MA, USA) for insemination after surgery. Each recipient was inseminated with the two doses of semen.

Laparoscopic Transfers

In the first experiment, 15 mares were used for laparoscopic procedures. Each mare was restrained in stocks and an intravenous catheter was placed into the jugular vein. Mares were premedicated (detomidine HCl, 5mg, iv, and butorphanol tartrate, 5 mg,iv). A detomidine infusion was established using 1L of sodium chloride solution with 10 mg detomidine. The infusion rate was titrated to an effective dose for sedation and

pain management. Prior to the procedure, mares were administered flunixin meglumine (1.1 mg/kg, iv; Prevail, MWI, Meridian, ID) and Procaine Penicillin G (35 ml, im)

The contralateral flank to the aspiration site was clipped 10 cm cranial to the last rib to 10 cm caudal to the tuber coxae and from 10 cm proximal to the tuber coxae to end of the last rib. The surgical site was aseptically prepared with alternating scrubs of povidone-iodine and alcohol. Three local blocks were performed using 10 ml of 2% lidocaine HCl at the sites for cannula placement. A 15-mm incision was made through the skin, subcutaneous tissues and the external abdominal oblique muscle at each of the portal sites. The first portal is midway between the last rib and the base of the tuber coxae. An insufflation cannula was placed through the incision and into the peritoneal cavity, and the trocar was removed to check for negative pressure in the peritoneum. The cannula was attached to a CO_2 insufflator, and the abdomen was insufflated at a rate of 3 L/min until a pressure of 15 mm Hg was attained. After a pneumoperitoneum was established, two additional incisions were made. The second portal was 5 cm proximal to the first portal and just caudal to the last rib and cranial to the first portal. The third portal was 10 cm distal to the first portal site. A laparoscopic cannula was introduced into the center portal; the trocar was removed and a10-mm diameter, 54-cm long, 30 rigid laparoscope was introduced into the abdomen. A light source and video camera were attached to the laparoscope. The peritoneal cavity was briefly explored, and the uterine horn, ovary and oviduct were identified. The laparoscope was then relocated to the upper most portal. Cannulas were placed in the middle and third portal sites and a set of Babcock atraumatic grasping forceps were introduced into both portals. The forceps were manipulated to grasp the oviduct. The ventral grasping forcep was removed, and the

appropriate catheter was introduced. The grasping forcep was used to facilitate passage of the catheter into the oviduct. Once the oviduct had been catheterized, the oocytes were expelled. The proximal and distal most cannulas were removed and the middle cannulas remained to facilitate expulsion of CO_2. After sufficient CO_2 was removed the last cannula was removed. The skin incisions were closed using a single cruciate suture per incision using a non absorbable suture (3 Braunamid).

Three different catheter systems were utilized to identify the best method for ease of use, transfer and catheterization: 1) Cassou embryo transfer gun (n=7) (18 inches, 0.25 ml, PETs Inc., Canton, TX), 2) Cook laparoscopic catheter (n=3) (Mars Laparoscopic Gift Catheter, 5 French, 30 cm, Cook IVF, Australia) and 3) MILA human laparoscopic catheter (n=7) (Laparoscopic Prototype, MILA International Inc., Erlanger, KY). Oocytes were preloaded into the appropriate catheter after the oviduct had been successfully grasped.

In the second experiment, 15 mares were used for oocyte collections and laparoscopic surgeries. Sedation was changed to intermittent intravenous xylazine for sedation, instead of detomidine infusions. The surgical approach was similar, with the exception that for mares with body condition scores of >7, a fourth portal was utilized, 5 cm proximal to the first portal and in the last intercostal space. A single catheter type, disposable embryo transfer gun using a 0.25-ml straw (Veterinary Concepts, USA) was used.

Postsurgical Procedures

Mares were inseminated approximately 4 h after surgery with the cooled does of semen and returned to the dry lot after full recovery from anesthesia. The uterus of each

mare was ultrasounded the following day to check for the presence of uterine fluid. Pregnancy examinations were performed on days 12, 14 and 16 after oocyte transfer. If a pregnancy was detected, the pregnancy was observed until day 25 to determine presence of an embryo proper. After an embryo proper was identified, the embryonic vesicle was manually disrupted, and the mare received prostaglandin (Estrumate, 250 μg/ml cloprostenol sodium, $PGF_{2\alpha}$ analogue, administered 1 ml im, repeated for two days, Schering, Kenilworth, NJ, USA) to induce luteolysis.

Results

In the preliminary experiment, 17 surgeries were attempted. For 12 attempts, the catheter appeared to be successfully manipulated into the oviduct. The five failed transfers were caused by oviductal cysts blocking visualization, altered camera placement and inability to visualize oviduct, bleeding from muscle or from splenic capsule, loss of the oocytes, and failure to catheterize the oviduct. Pregnancies were detected in three recipients; two mares had two embryonic vesicles. All embryonic vesicles developed an embryo proper. Two of the pregnancies resulted from use of the MILA system and the third was from the use of the embryo transfer gun. No pregnancies were obtained using the Cook catheter.

In the second experiment, five surgeries were attempted, and four resulted in successful manipulation and passage of the catheter into the oviduct. The one failed surgery was due to inadequate insufflation of the abdomen. Pregnancies were detected in two mares; one mare had two embryonic vesicles.

Discussion

Oocyte transfer has become an option for mares that are unsuccessful embryo donors. However, the standard flank laparotomy requires longer recuperation time, more stall rest and longer dosing of antibiotic. Traditionally laparotomy procedures are associated with higher pain levels than minimal invasive approaches such as laparoscopy. Laparoscopic techniques have been used quite effectively for a variety of procedures, such as cryptorchid castrations, granulosa cell tumor removal, tubal ligation and nephrosplenic space ablation. These experiments, although preliminary, did identify a portal arrangement that greatly facilitated catheter placement. The catheter systems will need to be further evaluated for ease of use. Difficulties identified were the length of the catheter, the ease of expulsion of oocytes, and the duration of oocytes remaining in catheter.