THESIS

CHARACTERIZATION OF SELECTED GENE EXPRESSION PATTERNS AS POTENTIAL MARKERS FOR OOCYTE QUALITY IN YOUNG VERSUS OLD MARES

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY BERNARDO DE LIMA RODRIGUES ENTITLED "CHARACTERIZATION OF SELECTED GENE EXPRESSION PATTERNS AS POTENTIAL MARKERS FOR OOCYTE QUALITY IN YOUNG VERSUS OLD MARES" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

CHARACTERIZATION OF SELECTED GENE EXPRESSION PATTERNS AS POTENTIAL MARKERS FOR OOCYTE QUALITY IN YOUNG VERSUS OLD MARES

As a female ages a series of alterations in normal physiology take place, during this process fecundity decreases. The reproductive system starts to shut down as a consequence of hormonal, histological and anatomical changes, and in the middle of this process, playing a critical role, is the oocyte. As in women, fertility decreases with aging in mares. Recently the mare has been suggested as a promising model to study age-related infertility in women due to similarities in the reproductive cycle and similar age-associated reproductive changes.

During the last decades the use of assisted reproductive techniques (ART) in human and veterinary medicine to treat infertility has increased. Unfortunately, ART can only partially compensate for declining fertility– particularly the age related decline in fertility. Therefore, we need to understand the mechanisms involved in age-associated infertility and improve both the diagnostic tools and the techniques currently used in ART. In that regard the identification of reliable oocyte quality markers is of great interest, specifically of extrinsic markers in follicular cells and

follicular fluid (FF). Follistatin (*FST*) and anti-müllerian hormone (*AMH*) have been suggested as potential oocyte quality markers. In addition, the rate of apoptosis in follicular cells has also been suggested to be a good indicator of oocyte quality

In this study, our goal was to use the young and old mare model to obtain competent and incompetent oocytes, respectively, to try and elucidate the involvement of apoptosis of follicular cells and/or of the oocyte in the determination of oocyte quality. Oocytes, follicular fluid, granulosa and cumulus cells were collected through transvaginal follicular aspirations from young (4-10 years) and old (>20 years) mares. Preovulatory follicles were aspirated 30-36 h post induction of follicular maturation, which was performed by administration (i.v) of a combination of deslorelin and hCG when the biggest follicle reached 35 mm. We used real time PCR to examine expression of pro-apoptotic (*CASP2, CASP3*) and pro-survival (*XIAP*) genes, as well as of *FST* and *AMH* expression in these cell types. In addition we measured androgens and estrogens in FF and calculated the androgens to estrogens ratio to assess follicular atresia. We also sought to determine FF concentrations of FST and AMH, and relate it to oocyte quality.

There was no difference in *CASP3* expression levels in granulosa and cumulus cells between the two age groups. In addition, there was no difference in *CASP2* and *CASP3* mRNA expression in oocytes from young and old mares. *XIAP* mRNA levels were expressed 3.3 fold higher in oocytes from young when compared to old mares, and there was a tendency for *XIAP* to be more highly expressed in granulosa cells of young mares. In contrast, the levels of *XIAP* mRNA in cumulus cells were 1.46 fold higher in old when compared to young mares. There was no difference in the

expression levels of *AMH* in granulosa cells between young and old mares, but in cumulus cells there was a tendency for *AMH* to be higher expressed in cells from old vs. young mares. Unfortunately we were not able to analyze AMH FF concentrations. *FST* mRNA levels in oocytes were similar between the age groups, but FST concentrations in FF of preovulatory follicles from young mares (197 ± 16.7 ng/mL) were higher (p=0.02) than in FF from old (153.3 ± 22.7 ng/mL) mares. In both age groups FST FF concentrations in preovulatory follicles significantly decreased when compared to mid-estrus and post-deviation follicles.

In conclusion, we believe that our data suggest that FST follicular fluid levels can be a non-invasive marker to assess oocyte quality in the horse, and that FST levels decrease in preovulatory follicles of the horse. In addition, expression levels of caspase-3 in follicular cells, and caspases 3 and 2 in the oocyte, does not seem to be involved in the mechanism of fertility loss in the old mare. Finally, *XIAP* mRNA levels may be important for oocyte quality in the horse.

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I dedicate this work to my family, especially to my nieces Mariana and Camila, and my nephew Pedro.

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CHAPTER I: INTRODUCTION

As a female ages a series of alterations in normal physiology take place, and it is long known that during this process fecundity decreases. The reproductive system starts to shut down as a consequence of hormonal, histological and anatomical changes, and in the middle of this process, playing a critical role, is the oocyte [109; 133; 162].

Many studies have demonstrated a detrimental effect of maternal aging on fertility in several species, including humans and horses, that seems to be strongly related to oocyte quality [109; 18]. The loss in oocyte quality that occurs with aging is a multifactorial process, involving different factors at different stages of the cycle. Although great efforts have been put forth to understand and treat age-related infertility, the complete elucidation of this condition has not been achieved.

Fertility decreases with aging in mares, starting to decline in the early teen years, and reproductive senescence usually is reached after twenty years of age [20]. Recently the mare has been suggested as a promising model to study age-related infertility in women due to similarities in the reproductive cycle and similar age-associated reproductive changes [54; 20]. Also the lifespan of the mare is approximately 25 years and exposure of these animals to environmental factors is similar to humans [20]. Therefore any elucidations of the mechanism of age-related infertility and advances in assisted reproductive techniques (ART) in the mare, besides greatly contributing to

equine reproduction, can also be used to validate and improve studies performed in other species, more specifically humans.

In modern society, women are displaying an increased tendency to delay pregnancy until later ages. Many women wait until after their peak fertility to attempt to conceive and are therefore unsuccessful. A couple that fails to conceive after 12 months of unprotected intercourse is classified as subfertile [41]. Many of these couples will resort to ART, which are only able to compensate for the decrease in fertility to some extent [13]. The growing utilization of ART has led to an aid to develop new techniques and improve the ones currently being used. An area of growing interest is the identification of fertility markers. Although the success of embryo transfer, as positive pregnancy results and offspring viability, remain the ultimate way to define oocyte quality [104], it would be a great advantage to be able to discern competent from incompetent oocytes prior to the application of *in vitro* fertilization and transfer procedures.

The traditional methods of oocyte evaluation are very subjective and, therefore, controversial because they rely upon using morphological characteristics to determine quality and are based on the assessment of cumulus–oocyte-complexes, polar bodies, spindles, and even follicular and ovarian morphology [156]. In order to establish reliable cellular and molecular markers of oocyte quality, considerable research has been done and many potential markers proposed. Specifically, much effort has been directed towards the identification of extrinsic predictors of oocyte quality, looking at surrounding tissues such as follicular cells and follicular fluid (FF). Recently, follistatin (FST) and anti-müllerian hormone (AMH) have been implicated as markers of oocyte quality. AMH

and FST FF concentrations in humans have been suggested to be positively correlated with oocyte quality [50; 44; 141]. In addition, *FST* mRNA levels in bovine oocytes are directly related to competence, and maternal derived *FST* is suggested to play a role in early embryonic development [114; 93].

Another area of interest is the relationship between apoptosis of follicular cells and the quality of the oocyte. Several studies have been conducted investigating this relationship, yielding conflicting results. One theory is that because of the intensive cross-talk between the oocyte and follicular somatic cells, the health of these cells is important for proper follicular and oocyte development [115]. Consequently, apoptosis of follicular cells is detrimental to the oocyte [67; 68; 23]. An alternate theory states that slightly atretic follicles contain more mature oocytes, and that the number of apoptotic follicular cells is positively correlated with oocyte maturity and quality [29; 57; 66].

Caspases play a central role in apoptosis in all cell types [75] and although levels of the active protein are more correlated with the occurrence of apoptosis, it has been reported that mRNA levels in cells are also related to the occurrence of apoptosis [48; 10; 99]. Caspase-3 is the primary caspase involved in granulosa cell apoptosis and has been suggested to have a central role during follicular atresia [45]. However, even though caspase-3 (CASP3) is expressed in the oocyte, caspase-2 (CASP2) is believed to be the main caspase involved in oocyte attrition [123]. X-linked inhibitor of apoptosis (XIAP) is a member of the inhibitors of apoptosis (IAP) family and prevents apoptosis by blocking caspases, more specifically caspase 3, 7 and 9 [25; 159]. XIAP mRNA levels in granulosa cells have been suggested to be related to follicular health [96; 2].

Therefore we hypothesized that: 1) Apoptosis of follicular cells and/or of the oocyte disrupts follicle development and consequently compromises oocyte quality. A higher incidence of apoptosis of follicular and/or germ cells is one of the mechanisms involved in age related infertility, and consequently *CASP2* and *CASP3* expression will be higher in samples from old mares. In contrast *XIAP* will be higher expressed in samples from young mares; 2) FST is expressed in equine oocytes; *FST* mRNA expression in oocytes and protein levels in FF are positively correlated with oocyte quality and are higher in samples from young versus old mares; 3) *AMH* mRNA expression in granulosa and cumulus cells is positively correlated with oocyte quality, and is higher in samples from young versus old mares.

To test our hypotheses we collected oocytes, granulosa and cumulus cells and FF from young and old mares. Oocyte mRNA levels of *FST*, *XIAP*, *CASP2* and *CASP3* were analyzed using real time PCR. Granulosa and cumulus cells mRNA levels of *AMH*, *XIAP* and *CASP3* were also examined. FF concentrations of FST, androgens and estrogens were determined. In this study, our goal was to use the young and old mare model to obtain competent and incompetent oocytes, respectively, to try and elucidate the involvement of apoptosis of follicular cells and/or of the oocyte in the determination of oocyte quality. Moreover, we wanted to determine if the expression of pro-apoptotic (CASP2, CASP3) and pro-survival (XIAP) genes in these cell types could be used as potential oocyte quality markers. In addition, we sought to determine if *FST* was expressed in equine oocytes, and if *FST* mRNA levels were differentially expressed in young versus old mares. We also wanted to determine the concentration of FST in FF at different stages of follicular development, and verify if FST concentration in

preovulatory follicles could be used as an oocyte quality marker. Finally we analyzed the expression of *AMH* in equine granulosa and cumulus cells to determine potential differences in *AMH* mRNA expression in young vs. old mares, and also sought to validate an AMH ELISA assay to measure AMH concentrations in equine FF and serum.

CHAPTER II: REVIEW OF LITERATURE

Oocyte competence

Oocyte competence is defined as the ability of the oocyte to resume meiosis, cleave upon fertilization and develop into a normal embryo and healthy offspring. In order to successfully complete all these events, the oocyte has to undergo serial stages of maturation during folliculogenesis, including: meiotic, cytoplasmic, molecular and epigenetic maturation.

Mammalian oocytes are arrested at prophase I before induction of meiotic maturation, and maintenance of meiotic arrest is caused by high levels of cAMP [126]. Meiotic maturation is related to the cascade of events that will culminate in the resumption of meiosis and is induced either by the LH surge or the removal of the oocyte from the follicular environment [139]. Following either of these signals, maturation promoting factor is either synthesized and/or activated and the oocyte completes the first phase of meiosis and releases the first polar body [139]. At this point the oocyte is arrested at metaphase of meiosis II under the influence of cytostatic factor [139].

In the horse, as in most mammalian species, meiotic maturation is gradually acquired during follicular growth, more specifically after the follicle reaches 20 mm in diameter [66]. During follicular development as the oocyte grows it gains meiotic competence in three stages. First it acquires the capacity to undergo germinal vesicle

breakdown, then it becomes capable of progressing to metaphase I, and finally it gains the ability to progress to metaphase II [129].

Cytoplasmic maturation involves the events that will capacitate the oocyte to complete its development and includes an increase in the number and a reorganization of intracellular organelles, such as mitochondria, lysosomes, endoplasmic reticulum and the Golgi complex [157]. In addition, the oocyte accumulates calcium within vesicles that are required at fertilization [14] and also develops inositol 1,4, 5-trisphosphate (IP3) sensitivity by increasing the number of IP3-receptors in preparation for fertilization [40]. One of the first signs of cytoplasmic maturation in the oocyte is nucleolus condensation and ribosome depletion, representing the cessation of the preparation phase due to modification in the transcription and translation machinery [139].

Molecular maturation is related to the accumulation of mRNA, proteins and substrates that will be required during early embryonic development. It is believed that specific mRNA and proteins are produced and stored in the oocyte in the last few days before ovulation [89]. A good example are maternal effect genes (MEG), which are expressed preferentially in the oocyte during growth [28]. Their products (mRNAs, microRNAs and proteins) are critical for embryo development and are usually present in early embryos and degraded at the time of maternal embryonic transition (MET) [5].

MET consists of the shift from mRNAs and proteins stored in the oocyte to those actively produced by the embryo, after embryonic genome activation [5]. It is a crucial process that occurs gradually and varies between species, taking place at the 1- to 2-cell stage in mice [144], 4- to 8-cell stage in humans [144] and horses [12], and around the 8- to 16-cell stage in cows and sheep [152]. Therefore it is believed that molecular

maturation is the main intrinsic event during oocyte development that will determine its capacity to reach blastocyst stage and progress to a successful pregnancy [139].

Epigenetic maturation encompasses molecular and cellular alterations that occur during oocyte development and are related to gene expression but do not involve alterations in gene structure [14]. Some of the mechanisms involved in epigenetic processes include chromatin methylation and histone acetylation. Disturbances in follicular cell physiology impact the epigenetic quality of oocytes and can have effects on the success of fertilization, embryo development and pregnancy or health of the offspring [14]. Current hypotheses to explain the loss of oocyte quality in old females are based in epigenetic alterations that may arise from the physiological dysregulations that occur with aging. The production of an epigenetically competent oocyte is dependent on the follicular environment during germ cell development.

Oocyte growth and differentiation are usually coordinated with the development of the somatic components of the ovarian follicle, and specific changes in the oocyte occur at specific follicular stages [38]. The intimate association between the oocyte and its surrounding cells is critical for normal follicular growth and acquisition of oocyte competence [102]. Oogenesis and folliculogenesis are closely correlated and it is now believed that the oocyte controls follicular growth by regulating its microenvironment [39; 72]. The oocyte plays a role in promoting follicular growth and directing granulosa cell differentiation, for example, the oocyte is directly responsible for the establishment of the distinct cumulus granulosa cells lineage [53]. On the other hand, granulosa cells can influence various oocyte functions, including modulation of transcriptional activity [129]. Furthermore, cumulus cells directly influence oocyte development and are thought

to participate in oocyte meiotic arrest and resumption as well as in oocyte metabolism [104].

The communication between oocyte, mural granulosa and cumulus granulosa cells is bi-directional and occurs via paracrine signaling and gap-junctional exchange of small regulatory molecules [53]. Some of the paracrine oocyte-secreted factors include bone morphogenetic protein-6 (BMP-6), growth differentiation factor-9 (GDF-9), GDF-9B (or BMP-15), as well as other members of the transforming growth factor-β (TGF- β) superfamily [52]. These factors regulate granulosa cell growth and differentiation as well as cumulus cell expansion and ovulation [52]. The gap-junctional exchange of regulatory molecules occurs through trans-zonal cytoplasmic processes, present in cumulus cells, which penetrate through the zona pellucida and connect with the oocyte membrane [1]. Some of the molecules that are exchanged through this pathway include small regulatory molecules, ions, metabolites and amino acids that are necessary for oocyte development [52]. Large molecules are transported by receptor-mediated endocytosis [52]. Therefore the cross-talk between the oocyte and the surrounding follicular cells is essential for follicular development and ovulation of a competent oocyte, and therefore can have direct and indirect effects on fertility.

Fertility markers

The growing utilization of ART's in human and veterinary medicine has fueled efforts to continuously improve the available techniques, and improve pregnancy outcomes. Because of the influence oocyte quality has on fertility, the identification of reliable predictors of oocyte developmental competence is an important area of research.

Although pregnancy rates after embryo transfer and offspring viability remain the ultimate test of oocyte quality [104], it would be of great advantage to be able to use predictive parameters to discern competent from incompetent oocytes prior to the expensive and labor intensive procedures of *in vitro* fertilization (IVF) and embryo transfer.

The most traditional methods to evaluate oocyte quality are based on morphological characteristics and assessment of cumulus–oocyte-complexes, polar bodies, spindles, and follicular and ovarian morphology [156]; However these methods can be very subjective and therefore controversial [156]. In order to establish reliable cellular and molecular markers of oocyte quality, considerable research has been done and many potential intrinsic oocyte parameters identified, such as mitochondrial status, calcium, ATP or glutathione content and phosphodiesterase activity [156]. Despite the fact that these parameters can be used to evaluate quality of oocytes, some of them cannot be considered predictive and are too invasive [104].

The identification of reliable markers in surrounding tissues would be very beneficial, primarily because it would not hamper the use of the oocyte. Therefore considerable effort has been directed toward identifying extrinsic predictors of oocyte competence, looking at granulosa cells, cumulus cells and follicular fluid (FF). Some of the parameters evaluated include expression patterns of insulin-like growth factors and insulin-like growth factor binding proteins in FF, steroid concentrations in FF or serum, oxidative stress in FF and granulosa cells, leptin concentrations in FF or serum, and gene expression profiling of cumulus cells [156].

Apoptosis in cumulus and granulosa cells and its relationship to oocyte quality has been studied in different species and controversial results have been reported. While some investigators propose that the number of apoptotic granulosa cells is negatively correlated with oocyte competence, some state that apoptosis of follicular cells is positively correlated with the quality of the oocyte. In addition, members of the TGF-β superfamily and its association with oocyte quality have been investigated. Recent studies suggested intrafollicular levels of AMH as a promising marker for oocyte quality in human IVF programs [44; 141]. *FST* mRNA levels in bovine oocytes have been reported to be positively correlated with the oocyte developmental competence, and maternal *FST* was proposed to be playing an important role in early embryonic development [114; 93].

Due to the difficulty in obtaining large numbers of equine oocytes to perform experimental studies there is limited amount of information regarding oocyte competence and fertility markers available in the horse. In addition, successful *in vitro* fertilization techniques (more specifically ICSI) in the horse have only become available fairly recently. Therefore most of the data obtained to date pertains to oocyte meiotic maturation, which does not always represent developmental competence. Ginther and collaborators reported higher levels of progesterone and insulin growth factor-1 and lower levels of estradiol in FF from mature oocytes versus immature (based on the presence or absence of the first polar body, respectively) [57]. In addition, they also reported higher number of apoptotic mural granulosa cells derived from follicles containing mature oocytes versus immature oocytes [57]. Dell'Aquilla and collaborators, utilizing oocytes aspirated from different size follicles from ovaries obtained in abattoirs, reported a positive correlation between granulosa cell apoptosis and oocyte meiotic

competence [29]. In contrast, Pedersen and collaborators demonstrated that the quality of the oocyte is directly related to the health of surrounding granulosa cells [115].

Currently the main criteria utilized to classify equine oocytes is based on morphological assessments of the follicle during follicular growth and just prior to follicular aspiration, as well as morphological assessments of the oocyte and surrounding cells upon collection. It is suggested that in order to maximize *in vitro* maturation and fertilization results, one should select equine oocytes based on the presence of an expanded cumulus and an unevenly colored cytoplasm [65]. However, these techniques are highly subjective and do not always correlate with oocyte competence. Therefore, due to the small amount of information and the conflicting results obtained in the horse to date, the identification of a reliable marker of oocyte developmental competence is yet to be achieved.

Anti-Müllerian Hormone

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a homodimer glycoprotein of 140 KDa [122; 15], a member of the TGF-β superfamily [97], and is expressed only in the gonads [92]. Members of the TGF- β superfamily signal through a serine/threonine kinase receptor complex that includes specific type II receptors and more general type I receptors. [155]. The AMH type II receptor is highly specific and expressed by AMH target organs, namely the Müllerian ducts and the gonads of both sexes [13]. The identity of the type I receptor is still not clear, and three type I receptors of Bone Morphogenic Proteins, activin like receptor (ALK) 2, Alk3, Alk6, may transduce AMH signals [31]. For example, ALK2 is involved

in Müllerian duct regression [153]. This crossover is likely due to the fact that AMH uses the same pathway as the BMP family, mediated through the downstream signaling molecules Smad1, Smad5, and Smad8 [155]

AMH is encoded by a 2750 bp gene divided into five exons [77]. The 3′ part of the fifth exon codes for the bioactive part of the molecule and it is extremely rich in guanine and cytosine residues [77]. The AMH gene is highly conserved and has been cloned in the human, bovine, mouse, rat, pig, chick and alligator [125]. AMH expression is found both in testes and ovaries [143; 35]. However, during fetal development, AMH expression is restricted to the testis [154]. AMH produced by the Sertoli cells during fetal development causes regression of the Müllerian ducts that would otherwise differentiate into the oviducts, uterus and upper part of the vagina [92]. After birth, sexually dimorphic expression of AMH is lost, and is also expressed in granulosa cells of growing follicles in the ovary, beginning at the primary follicle stage [155].

Besides its role in sex differentiation, AMH appears to also regulate subsequent testis function in the prepubertal male, as it suppresses Leydig cell differentiation and steroidogenesis [124]. In the male, testicular AMH secretion is maintained at high levels until puberty, at which time an increase in androgens and the initiation of meiosis appears to reduce its expression [125]. Also at this time concentrations of AMH in seminal plasma become higher than in blood due to a switch in the direction of secretion from the basal to the ad-luminal compartment of Sertoli cells [125].

Abnormalities in expression of AMH or its receptors have been previously associated with pseudo hermaphrodites, as well as Sertoli cell tumors in humans and animals [3]. Therefore serum AMH concentrations may be useful markers for these

conditions [3]. In the horse, Ball and collaborators recently demonstrated that AMH was strongly expressed in Sertoli cells in fetal, neonatal and prepubertal equine testis, but not in normal adult testis [3]. Also, they reported AMH expression in equine Sertoli cell tumors and in gonads collected from male pseudo hermaphrodites, suggesting that AMH may be useful in characterizing testis tumors and ovotestis gonads in the horse.

In the ovary AMH is first expressed in granulosa cells from primary follicles that are first found in mice on postnatal day 3 or 4, and in the human fetus after 36 weeks of gestation [155]. Expression is similar between the two species and persists in growing follicles, reaching maximal levels in pre-antral and small antral follicles, diminishing as the follicles grow and becoming almost undetectable in preovulatory follicles [80]. In humans at the later stages of follicular development expression becomes higher in cumulus cells and decreases in mural granulosa cells [158]. Follicles showing signs of atresia also have decreased or no AMH expression [35]. Expression is completely absent in corpora lutea, and is not evident in primordial follicles, theca cells, oocytes or the interstitium [35].

In the horse, a recent study reported AMH immunolabeling of follicles that consisted of more than one layer of granulosa cells, increasing in antral follicles, but with decreasing intensity in larger (>30 mm) follicles [4]. Follicles undergoing early atresia had minimal immunodetectable AMH and atretic follicles and corpora lutea had no immunodetectable AMH. This pattern of immunoreactivity is similar to that previously reported for other mammals, such as the sheep [16] cow [105], mouse and human [80]. Bioactive AMH was also detected in the serum of mares, based on its ability to inhibit Müllerian duct development in the fetal rat [4].

In vivo and *in vitro* studies indicate that AMH is functionally related to the regulation of both the number of growing follicles and their selection for ovulation [155]. Using the AMH null mouse model, Durlinger and collaborators demonstrated that animals lacking AMH displayed increased recruitment from the primordial follicle pool [32], such that the higher rate of primordial follicle recruitment led to premature exhaustion of the follicular reserve, causing early cessation of ovulation in ageing animals [33]. In a subsequent study, using 2 day old AMH null mice ovaries cultured in the presence of AMH, it was demonstrated that AMH had a direct effect on inhibiting primordial follicle recruitment [36]. Specifically, after 2 days of exposure to AMH, growing follicles were reduced by approximately 50% [36].

It was hypothesized that in the absence of AMH, follicles are more sensitive to follicle stimulating hormone (FSH) since AMH null female mice had lower FSH levels but higher number of growing follicles when compared to wild type animals [32]. The effect of AMH on FSH sensitivity was later confirmed, by *in vitro* studies showing that FSH stimulated pre-antral follicle growth in culture was attenuated by AMH [34]. Moreover, in immature AMH null female mice more follicles started to grow under the influence of exogenous FSH when compared to wild type mice [34]. Therefore, AMH may be involved in determining the responsiveness of the follicle to FSH during cyclic recruitment. This is also supported by the differential expression of AMH observed at estrus in non-atretic large pre-antral and small antral follicles [36]. Follicles with low AMH expression would be more sensitive to FSH, and thus more likely to be selected for continued growth [36].

This AMH-induced inhibition in follicle growth was considered to be the result of reduced granulosa cell proliferation [34] and is consistent with previous studies in which it was shown that exogenous AMH reduced aromatase expression and the number of LH receptors in cultured granulosa cells [155]. A recent study demonstrated that intrafollicular concentrations of AMH in small human antral follicles are inversely correlated to mRNA expression of the aromatase enzyme (CYP19) and estradiol concentration in FF [110]. These results are consistent with previous *in vitro* studies using human granulosa cells [60], bovine follicles [105; 127] and fetal sheep ovaries [151]. These results also suggest that AMH may play a major role prior to selection of the dominant follicle and possibly contributes to maintaining low intrafollicular estradiol production in the pool of growing follicles when compared to preovulatory follicles. Such an arrangement would, presumably, not interfere with the important interplay between the pituitary and the dominant follicle [110].

A study using co-cultures of oocytes and granulosa cells, demonstrated that *AMH* mRNA levels in granulosa cells of early and late pre-antral follicles, and cumulus cells of preovulatory follicles are up-regulated by the oocyte in a stage-specific fashion [131]. Thus, controlling AMH expression, growing oocytes may regulate follicle growth by decreasing the responsiveness of growing follicles to FSH [131].

To better understand the mechanisms by which AMH may exert its functions on follicular growth regulation an *in vitro* study of rat ovaries examined the pattern of gene expression in AMH treated ovaries and the interactions between AMH and an oocyte-derived growth factor (bFGF), a granulosa-derived growth factor (KITL), and a precursor theca-derived growth factor (KGF). AMH inhibited the stimulatory actions of KITL,

bFGF, and KGF; and altered the expression of 707 genes, decreasing the expression of stimulatory factors, increasing the expression of inhibitory factors and regulating cellular pathways (TGFβ signaling) resulting in the inhibition of primordial follicle development [111]. AMH was also shown to oppose endothelial growth factor-induced proliferation of cultured granulosa-luteal cells [81].

The release of AMH from ovarian granulosa cells, mainly by small antral follicles, leads to measurable serum levels which are proportional to the number of developing follicles in the ovaries [13]. Serum AMH levels decline with increasing age in healthy mice [80] and women [27], and directly correlate with declining numbers of growing and primordial follicles in both species [80]; becoming undetectable in menopausal women [27].

For several reasons serum levels of AMH may be a reliable marker of ovarian aging in humans [80]. 1) Serum AMH correlates strongly with antral follicle count (AFC) [27; 149]; 2) it is a good indicator of ovarian response to controlled ovarian hyperstimulation [43]; 3) it remains relatively constant during the menstrual cycle [90], and is not influenced by gonadotropic status [149]. In the bovine, serum AMH is also highly correlated with AFC and the number of morphologically healthy oocytes and follicles [73] and is also a reliable indicator of individual responses to superovulatory treatments [127].

Studies have also correlated intrafollicular levels of AMH with follicle quality [46] and as predictors of oocyte fertilization [141] and embryo implantation [44] following *in vitro* fertilization (IVF) treatments in women. A prospective study using 118 infertile IVF patients, concluded that FF AMH concentrations are strongly correlated and

positively associated with embryo implantation, and suggested that AMH concentrations in FF reflect granulosa cell function and oocyte health [44]. In addition, Takahashi and collaborators compared values of AMH in FF samples collected at the time of oocyte retrieval from 31 women undergoing IVF cycles [141]. They reported that FF levels of AMH were 3.42 times higher in the fertilized group than in the non-fertilized group, suggesting that oocytes are more likely to be fertilized when follicles contain high concentrations of AMH [141].

In women, serum AMH levels initially have been used as a marker for granulosa cell tumors, enabling the detection of recurrence of these tumors at an earlier stage [13]. Serum AMH levels can also reflect subtle damage to ovaries and may be the most appropriate test to determine the extent of ovarian damage caused by chemotherapy or ovarian surgery [13]. Moreover, human follicular cysts exhibit high intrafollicular concentration of AMH, and production of AMH per granulosa cell is 75 times higher in anovulatory polycystic ovarian syndrome (PCOS) as compared with normal ovaries [116]. Finally in the horse, serum AMH recently has been suggested as a potential marker for granulosa cell tumors [4], but no work has been done to address its role in folliculogenesis, or as a possible marker of ovarian ageing and oocyte quality.

Follistatin

Activins, inhibins and follistatin (FST) form a distinct subfamily of the TGF-β superfamily, and are important to a number of reproductive and non-reproductive functions [121]. Activins and inhibins are structurally related, they are both dimeric proteins, with inhibins being made of α-β dimers, and activins made up of β-β dimers

[121]. There are two known forms of inhibin (α-βA, α-βB) and three of activin (βA-βA, βB-βB, βA-βB) [121]. FST is structurally unrelated to the activins and inhibins, but binds with high affinity to the β-subunits [63]. Although activin is its highest affinity ligand, FST also binds and inhibits many other TGF- β family members, including inhibin, and several bone morphogenic proteins (BMPs) [63].

FST was discovered in 1987 by two independent groups working with FF of bovine [128] and swine [147], due to its ability to suppress hypophysial FSH independent from inhibin. This ability to suppress FSH later was discovered to be due to its activin-binding activity [107]. FST is a cysteine rich monomeric glycoprotein, encoded by a single gene highly conserved across species (>97%) [47], but exists in multiple isoforms due to alternate mRNA splicing [160]. There are three major isoforms of follistatin, originated by alternative splicing and proteolytic processing [63]. These major isoforms undergo further post translation modifications to produce the six different isoforms that have been identified in ovarian FF of pigs and cows [58]. Recently, a separate gene product, follistatin-like 3 (FSTL3) or follistatin-related protein (FRSP), has been isolated and is functionally and structurally related to FST, but its biological role is still not clear [121].

The functional significance of these different isoforms is not known. Although all seem to inhibit association of activin with its receptors, it is thought that under certain circumstances some might serve to enhance activin presentation to its cell surface cognate receptor(s) [83]. Some isoforms of FST have a high affinity for cell surface proteoglycans, and might serve to sequester activin on the cell surface while the other forms bind and neutralize activin in blood and extracellular fluid [83].

Activin and other members of the TGF-β superfamily are multifunctional hormones, and the nature of their effects depends on the cellular context in which they are applied [98]. They are involved in regulating a wide range of cellular events, such as differentiation, repair, cell adhesion and apoptosis [98]. Their signaling involves a family of membrane receptor protein kinases and a family of receptor substrates, the SMAD proteins, which will localize in the nucleus and act as transcription factors [98]. FST binds to activin with a similar or higher affinity of that between activin and its receptor, and the bound complex is composed of one FST molecule bound to each of the two activin β-subunits [76]. Although each tissue should be considered separately, generally FST and inhibin will exert antagonistic actions to those of activin. FST is encountered in a variety of tissues, including ovaries, testis, uterus, placenta, pituitary, liver, and endothelial cells [121]. However, FST plays different roles in each system and is involved in several physiological pathways, from the regulation of FSH release in the pituitary [7] and follicular development in the ovary [47], to inflammatory processes throughout the body [87].

In the anterior pituitary gland, locally produced activins act in a paracrine and autocrine manner to stimulate the differential production of FSH from gonadotropes [7]. FST, which is also produced in the pituitary gland, binds activin and modulates its actions. In addition, inhibins, produced by the gonads act in an endocrine manner and are involved in a feedback loop system between the gonads and the pituitary [7]. A fine balance between these factors is essential to maintain a normal reproductive cycle [7]. Due to the importance of FSH on testis physiology, the modulation of its secretion by inhibin, activin and FST influence testicular function. Inhibin most likely affects the testis

through a feedback loop involving the pituitary [88]. While activin and FST have local actions in the pituitary, they are both produced by a large number of cell-types in the testis, and are involved in modulation of androgen production, proliferation of Sertoli and germ cells, and affect the structural and functional features of mitochondria within germ cells [88].

In the mammalian ovary, activins, FST and inhibins synthesized by follicular granulosa cells modulate follicular growth, gonadotropin responsiveness, steroidogenesis, oocyte maturation, ovulation, and corpus luteum function [83]. Activin is proposed to stimulate granulosa cell proliferation in developing follicles and upregulates FSH receptors and FSH-induced aromatase activity, as well as delaying luteinization and atresia of large antral follicles and enhances oocyte maturation [58]. FST, as an activin-binding protein, attenuates the actions of activin and some of the BMPs [58]; although actions outside of its activin-binding activities are also considered.

Activins, inhibins and FST activity is tightly regulated during folliculogenesis, underscoring their importance for follicular development; a regulatory feedback between the oocyte and follicular cells is probable, since the oocyte produces an unknown factor that stimulates mural and granulosa cells to increase production of these proteins [121]. Granulosa cell derived FST increases with preovulatory follicular development in the human and the bovine, but decreases in the sheep [161]. FST production by rat and bovine granulosa cells is stimulated by FSH, but not LH, in vitro [64]. FST is not expressed by mouse oocytes, but is expressed by human [138] and bovine oocytes [114].

During follicular maturation in humans, the intra-ovarian microenvironment changes from a primarily activin-B dominant to an inhibin-A/FST dominant environment

[161]. In addition, free FST concentrations in FF increase with both size and maturity of dominant follicles, and do not differ between atretic and healthy follicles [161]. The same pattern is observed in cattle where intrafollicular concentrations of FST and granulosa cell *FST* mRNA levels increase as the follicles develops and are higher in dominant versus subordinate follicles; however in contrast to humans atretic follicles, bovine atretic follicles have lower *FST* mRNA levels when compared to healthy follicles [58]. Interestingly, *FST* mRNA levels in granulosa cells of sheep seem to be reduced in preovulatory dominant follicles [146].

Mice overexpressing FST present extensive reproductive defects, such as decreased testis size and arrest in spermatogenesis in males, and a block in folliculogenesis in females [59]. Knockout mice are not viable due to multiple defects but conditional knockout females develop fertility defects, including reduced litter numbers and litter sizes, and in severe cases infertility, due to reduced numbers of ovarian follicles, ovulation and fertilization defects, elevated levels of serum FSH and LH [76].

In 1999, the cDNA encoding equine FST was isolated from an equine ovarian cDNA library, and *FST* mRNA was detected in uteroplacental tissues, follicles and corpora lutea [140]. In situ hybridization analysis indicated that in the follicle, *FST* mRNA was expressed exclusively in granulosa cells [140]. Ginther and collaborators reported a mean FST concentration in the FF of mares, in the range of 350 ng/mL and 180 ng/mL for the biggest and second biggest follicle respectively, at the time of deviation [56]. No further work has been done to determine *FST* mRNA levels in granulosa cells or the oocyte during follicle development or FST FF concentration after deviation.

In vitro studies in humans and cows have demonstrated that both activin and inhibin, and consequently FST, can influence maturation of the oocyte. Therefore the regulation of these proteins during follicular development might influence the coordinated process of oocyte and follicle maturation [50]. A study examining FF levels from 109 follicle aspirates from 31 IVF human patients, reported that FF levels of both FST and progesterone were higher in follicles with mature oocytes [50]. Recent studies in bovine have established a positive association of *FST* mRNA abundance in oocytes with developmental competence [114]. In a subsequent study, it was demonstrated that decreasing *FST* mRNA expression in bovine oocytes through injections of interfering RNA (siRNA), leads to a reduction in the proportion of embryos reaching the 8-16 cell stage and percentage of blastocyst development [93]. Subsequent studies also showed that exogenous FST supplementation during early stages of in vitro bovine embryo development (before embryonic genome activation) can reduce time to first cleavage, increase proportion of embryos developing to the blastocyst stage, and increase trophectoderm cell numbers [93]. These results suggest a potential role for oocyte maternal derived FST in bovine early embryonic development. This role has yet to be determined in the equine oocyte.

Apoptosis in the ovary

Apoptosis is a tightly regulated and highly conserved mechanism of cell death that is critical for tissue development and homeostasis [78]. Apoptosis is crucial for embryonic development [79], particularly development of the nervous system, and for both the development and functioning of the immune system [61]. Dysregulation of

apoptosis can lead to a series of pathological conditions. Specifically, excessive apoptosis is associated with ischemic heart disease, stroke, neurodegenerative disease, sepsis and multiple organ dysfunction syndrome; while deficient apoptosis is associated with cancer, auto-immunity and viral infections [78]. In the ovary, abnormally high rates of cell death can negatively affect fertility [75].

Apoptosis usually affects single cells causing minimal harm to nearby cells. This is distinct from necrosis, which causes widespread inflammation and damage to surrounding tissues [69]. Morphological evaluation remains the standard tool for detection of apoptosis, and the ultrastructural features of apoptosis are the following: condensation of nuclear chromatin, convolutions of the nuclear and cellular outlines, fragmentation of the cell and formation of apoptotic bodies, which will be phagocytized by macrophages [71]. However, other techniques, including DNA agarose gel electrophoresis, flow cytometry, and terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling and in situ end labeling, are also useful in detecting apoptosis [71].

Apoptosis is triggered by two general mechanisms. The first is related to the binding of "death molecules" to cell surface receptors (extrinsic pathways), including Fas and Fas ligand, tumor necrosis factor (TNF) receptor (TNFR), interferon (IFN) and TNF-related apoptosis-inducing ligand (TRAIL) receptors [100]. Binding of these ligands and receptors leads to the activation of death domains and caspase recruitment domains, which will activate adaptor proteins and procaspases causing the initiation of the caspase cascade and consequently apoptosis [100]. The second mechanism involves intracellular signals (intrinsic pathways), mediated either by mitochondria or the endoplasmic

reticulum (ER). The mitochondrial pathway involves changes in organelle permeability, causing the release of various factors that will lead to the activation of caspases and culminate with cell death [71]. In the mitochondria pathway, stress induces pro-apoptotic members of the B-cell lymphoma/leukemia-2 (Bcl-2) family of proteins that will cause cytochrome-c to leak from the mitochondria [71]. Cytochrome-c, in conjunction with apoptosis inducing factor (Apaf-1) will activate caspase-9, initiating the caspase cascade [71]. In the ER-mediated pathway a stress signal activates procaspase-12, which leads to the activation of caspase-9 and subsequently caspase-3, in a cytochrome-c independent manner [74]. In addition, recent data has shown that the ER can mediate apoptosis thru the mitochondria, and that caspase-2 is involved in the ER-mediated pathway [22].

Although all of the above described pathways can initiate cell death independently, evidence indicates important cross-talk between them [Jiang et al., 2003]. Central among these death pathways are the members of the caspase family; a family of cysteine proteases which are involved in both the initial and final stages of apoptosis in all cell types [75]. These proteins are synthesized as inactive zymogens (procaspases) and are activated by proteases induced by either pro-apoptotic or insufficient anti-apoptotic signals [37; 24]. After activation, caspases cleave a variety of intracellular polypeptides necessary for cellular homeostasis [37]. The mammalian caspase family includes 14 members, which can be classified into initiator caspases, such as caspase-8, 9 and 10, or effector caspases, like caspase-3, 6 and 7 [75]. Several caspases have been implicated in directly mediating apoptosis in the ovary, including caspases-2, 3, 9, 11, and 12 [75].

Procaspase activation plays a central role in mediating death pathways and these enzymes are the target of a series of pro-survival molecules that try to minimize or inhibit

unwanted apoptosis [74]. Regulation of caspase activity occurs at different levels, including gene transcription and inhibition of activation and enzymatic activity [113; 75]. Some of pro-survival molecules include members of the Bcl-2 family and the inhibitors of apoptosis (IAPs) family [74].

The IAPs are a family of cell survival proteins, which regulate cell death by binding to and inhibiting caspase activation and activity [30]; this family includes X-linked IAP (XIAP), human IAP-1 (HIAP-1), human IAP-2 (HIAP-2), neuronal apoptosis inhibitory protein (NAIP), Survivin, TS-IAP and Apollon/Bruce [134]. XIAP is the most potent inhibitor of apoptosis in the IAP family, and has been shown to function downstream of procaspase 9 cleavage as an inhibitor of both active caspase-9 and caspase-3 [25].

Apoptosis in the ovary was first reported by Flemming in 1885, when he described the hallmark alterations of apoptosis in granulosa cells during degeneration of antral follicles in rabbits. Since this initial observation, apoptosis has been identified as essential for many aspects of ovarian development and cyclic function, including oogenesis, folliculogenesis, luteolysis, oocyte selection and atresia [145]. Apoptosis starts during fetal life, when is localized to oocytes and is responsible for reducing the pool of ovarian follicles by at least two-thirds [71]. After birth, the depletion of the germ cell reserve can be initiated either by oocyte apoptosis or as a consequence of granulosa cells apoptosis [106]. Follicular atresia initiated by the oocyte is observed mainly in the early stages of follicle development (primordial to small preantral stages) [106], and it has been demonstrated to be genetically programmed and a significant contributor to the reduction of the oocyte reserve [119].

Follicular atresia initiated by apoptosis of granulosa cells occurs later in follicular development, and the fate of the follicle relies on the balance between factors promoting cell proliferation, growth, differentiation or cell death [74]. This process occurs due to granulosa cell death, followed by apoptosis of the theca cells, and is regulated by a series of factors, ranging from hormonal concentrations and interaction with receptors, intrafollicular paracrine and autocrine signaling, to proper communication between theca cells, granulosa cells and the oocyte.

Sufficient gonadotropin support is necessary for follicle development, survival and selection. Sex steroids are also suggested to play an important role in the regulation of apoptosis in the ovary. Specifically, estrogens prevent apoptosis while androgens induce [8]. Atretic follicles exhibit an increased androgen to estrogen ratio in FF [21; 103]. In addition, intrafollicular factors secreted by granulosa and theca cells, such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), transforming growth factor α and β (TGF- α, TGF- β) and TNF are also suggested to directly affect granulosa cell fate [24].

A common ovarian cell death pathway has been suggested, conserved across species and different cell types [145]. In the ovary Fas and Fas ligand are present in granulosa cells and are involved in the induction of apoptosis during follicular atresia; gonadotropin withdrawal allows for an increase in Fas and FasL levels and therefore apoptosis [74]. Furthermore, p53 protein, an antiproliferative factor, has been found to positively correlate with Fas and FasL content in granulosa cells, and it has been proposed that granulosa cell apoptosis is p53 dependent [82]. Specifically, p53 is inhibited by gonadotropins and overexpression of p53 causes Fas and FasL increase,

which leads to apoptosis [82]. The mitochondrial pathway also plays an important role in granulosa cell apoptosis, where balance between Bcl-2 and Bcl-2 associated X-protein (BAX) maybe the most important regulatory mechanism. Bcl-2 prevents apoptosis by blocking the release of cytochrome-c from the mitochondria [74]. Although, the involvement of the ER-mediated apoptotic pathway in the ovary remains to be elucidated, previous studies have associated this pathway with germ cell apoptosis and follicular atresia [117; 26].

Caspase-3 has been identified in the ovary of several species, and is considered the main effector caspase involved in granulosa cell apoptosis. Granulosa cell caspase-3 content increases in preovulatory follicles undergoing atresia, such that a direct correlation between caspase-3 expression, at both the mRNA and protein level, and apoptosis in granulosa cells of the rat ovary has been shown [48; 10; 99]. Moreover, granulosa cells from caspase-3 null mice are resistant to apoptosis induced by serum starvation [99]. Similarly, apoptotic cell death of both ovine and bovine luteal cells is correlated with an induction of *caspase-3* expression and or activity [24].

Caspase-3 is also expressed in the oocyte and has been shown to mediate germ cell death [42; 118; 120]. Cleaved active caspase-3 has been demonstrated in human oocytes that failed to cleave after fertilization [11] and active caspase-3 was also localized to both oocyte and granulosa cells of follicles undergoing atresia in mice [45]. However, the role of caspase-3 in oocyte apoptosis remains controversial. For example, despite the fact that caspase-3 is expressed in murine oocytes, knockout studies were unable to demonstrate that loss of caspase-3 affected either developmental or anti-cancer therapy-induced oocyte apoptosis [99].

Caspase-2 is the most conserved caspase across species and has the unique feature of acting as both as an initiator and an effector caspase, and appears to be necessary for the onset of apoptosis in response to multiple cellular insults [62]. Caspase-2 is derived from two different mRNA species resulting from alternative splicing, generating two proteins: caspase-2L (which induces cell death), and caspase-2S (which antagonizes cell death) [62]. Caspase-2 is expressed in both granulosa cells and oocytes in the ovary [48; 6], but seems to play an important role mainly in the oocyte [123], such that caspase-2 deficient female mice had normal rates of follicle atresia, but were born with an increased number of primordial oocytes due to decreased germ cell death during fetal development [6]. Furthermore, Bergeron and collaborators demonstrated that oocytes from adult caspase-2-deficient transgenic female mice are resistant to apoptosis induced by exposure to doxorubicin [6]. Recent studies with mouse oocytes indicate that insults that directly interfere with metabolic status require caspase-2 and caspase-3 as executioners [142]. However, in the event of caspase-2 and caspase-3 deficiencies, activation of caspase-12 leads to apoptosis [142]. Caspase-12 also appears to be a key player in the pathway(s) leading to oocyte death following induction of DNA damage in murine oocytes, and therefore may also be an important mediator of apoptosis in oocyte aging [142]. In humans, caspase-4 is the analog of murine caspase-12, and therefore is suggested to be involved in this mechanism [142].

Members of the IAP family also play an important role in the ovary. XIAP and HIAP-2 are expressed in rat granulosa and theca cells and appear to play a critical role in the control of follicular atresia [96]. XIAP can bind to and inhibit caspase-3, 7 and 9, but only HIAP-2 can inhibit caspase-2 [159]. In granulosa cells XIAP is positively correlated

with gonadotropin levels and seems to regulate apoptosis thru the phosphatidylinositol 3-kinase survival pathway [2]. Furthermore, XIAP overexpression results in reduced apoptosis, and its down regulation increases apoptosis [2]. Higher levels of *XIAP* mRNA were reported in bovine embryos derived from oocytes treated with leptin, which yielded higher blastocyst development rate and a decreased number of apoptotic cells, compared to non-treated oocytes [9]. In addition, NAIP has been suggested to contribute to granulosa cell survival and Matsumoto and collaborators demonstrated that NAIP down-regulation in granulosa cells indirectly affects oocyte development resulting in a decreased number of morphologically normal ovulated oocytes [101]. Finally, survivin may play a role as a maternal effect gene in the development of mouse oocytes and pre-implantation embryos [132]. Sato and collaborators demonstrated higher maternal *survivin* mRNA levels compared to zygotically transcribed mRNA levels in mouse pre-implantation embryos. Furthermore, *survivin* levels were lower in oocytes from aged mice [132].

Apoptosis in follicular and germ cells is proposed to directly affect fertility, but studies investigating the utility of apoptosis in follicular cells as a marker of fertility provided conflicting results. Dell'Aquilla and collaborators reported that, in the mare, granulosa cell apoptosis is related to cumulus expansion and oocyte meiotic competence [29]. In contrast, several reports have indicated that apoptosis in follicular cells negatively affect oocyte quality and consequently fertility. Host and collaborators demonstrated a significant increase in the number of apoptotic human cumulus cells in the pool of cells derived from immature oocytes compared with cumulus cells derived from mature oocytes, and suggested that apoptosis in follicles selected after hormonal

stimulation could be related to a lack of oocyte fertilization or low embryo quality in humans [67; 68]. Also, it has been demonstrated that the number of apoptotic granulosa cells is negatively correlated with pregnancy rates in women undergoing IVF treatments [108; 112; 23]. These findings are consistent with an earlier report by Pedersen and collaborators that correlated the health of granulosa cells with oocyte quality in the mare [115]. Additionally, apoptosis in bovine oocytes has been associated with disruption of oocyte competence [130]. Finally, Van Blerkom suggested that DNA fragmentation in oocytes could be a consequence of stress during ovarian follicle maturation [148], and that DNA fragmentation in oocytes may contribute to poor oocyte quality and lower fertility in aged mice [49].

CHAPTER III: MATERIALS AND METHODS

Animals

Nonlactating, light-horse mares were used for the experiments. Animals were assigned to two groups based on age: young (4-10 yrs; n=10), old (≥ 20 yrs; n=14). Mares were housed under natural light, in dry lots and fed alfalfa and grass hay. The experiments were performed during the breeding seasons of 2008 and 2009, from June to September, at latitude 40°N (Colorado, USA). All procedures were performed in accordance with and were approved by the Institutional Animal Care and Use Committee at Colorado State University.

Follicular aspirations and sample collection

During the breeding season of 2008, the reproductive tracts of mares were examined every other day by transrectal ultrasound (Aloka SSD, 500V; Aloka Science and Humanity, Wallingford, CT) with a 5-MHz linear transducer until the detection of a 25 mm follicle, when daily exams were performed until the detection of a follicle ≥ 35 mm, associated with endometrial folds and softening of the cervix. At this time a combination of 1.5 mg of deslorelin and 2000 IU of human chorionic gonadotropin (American Pharmacy Solution, Mobile, AL) was administered, i.v., to induce follicle and oocyte maturation. Ultrasound guided transvaginal follicular aspirations were performed [17] 35 (±2) hours post induction of maturation in order to collect FF samples, granulosa

cells, cumulus cells and oocytes. Briefly, for follicular aspirations a transvaginal plastic case containing the ultrasound probe and a double lumen 12 gauge needle (Cook Veterinary Products, New Buffalo, MI) was introduced in the vagina. The needle was introduced through the vaginal wall into the ovary, at which time the follicular contents were aspirated, and the follicle was flushed with 180 mLs of embryo flushing medium (EmCare™ Complete Embryo Solution; ICPbio Reproduction, Auckland, New Zealand) supplemented with 10 IU/mL of heparin (Calbiochem; La Jolla, CA) at 37°C.

The first 10 mLs of FF obtained, before the follicle was flushed with flushing medium, were collected for hormonal analysis. Immediately after follicle aspiration, FF samples were placed in an incubator at 38.5°C in an atmosphere of 6% CO_2 until oocyte identification, after which the FF was frozen and kept at -20°C. Different cell types were identified according to Carnevale and Mclellan [19]. Each cell type was isolated and processed as described below. A 10 µL aliquot of medium containing granulosa cells was collected and washed five times in phosphate buffered saline (PBS) supplemented with 0.02% polyvinyl alcohol (PVA). These cells were then stored in 20 µL of RNA Extraction Buffer (PicoPure™ RNA isolation kit, Arcturus, Sunnyvale, CA). After isolation of the cumulus-oocyte-complex, cumulus cells were removed mechanically under a stereomicroscope and processed as described for granulosa cells. The partially denuded oocyte was isolated and treated with hyaluronidase to remove any remaining surrounding cells, and then washed five times in PBS with PVA and placed in 10 µL of Extraction Buffer. Samples were stored at -80°C until RNA isolation.

In addition, during the breeding season of 2009, only FF samples were collected during three distinct time points of follicular maturation: post-deviation (25 mm; young

n=10, old n=7), mid-estrus (30 mm; young n=10, old n=10) and preovulatory follicles (30-36 h post induction of maturation, which was performed when the dominant follicle reached 35 mm; young n=10, old n=10). Follicular aspirations were performed as previously described.

RNA isolation

Total RNA was isolated from individual oocytes, granulosa cells and cumulus cells using the PicoPure™ RNA Isolation Kit (Arcturus, Sunnyvale, CA). Samples were treated with DNAse (Qiagen Inc, Valencia CA) and RNA concentration and purity from granulosa and cumulus cells was determined using nanodrop ND-1000 spectrophotometer. Oocyte RNA concentrations and integrity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara CA). RNA samples were stored at -80°C.

Reverse transcription of mRNAs

Complementary DNA (cDNA) was generated using the Qscript cDNA synthesis kit (Quanta Biosciences, Inc., Gaithersburg MD). Briefly, 5 µL of RNA (~50 ng/µL) plus qScript Reverse Transcriptase were combined with nuclease-free water and a 5X reaction mix containing optimized buffer, magnesium, dNTPs, and primers [oligo(dt)20 + random primers] . RNA was reverse transcribed by incubating the samples at 25°C for 10 minutes, 42°C for 60 minutes, and 95°C for 10 minutes. The cDNA was used immediately for real time PCR analysis.

Real-time PCR

The expression of anti-apoptotic (*XIAP*) and pro-apoptotic genes (*CASP2, CASP3*) and members of the TGF-β superfamily (*AMH, FST*) was examined using real-time PCR. *Tubulin* was used to normalize expression in oocytes, whereas *GAPDH* was used in granulosa and cumulus cells. Gene specific primers (Table 2.1) were designed using Primer3 design (http://frodo.wi.mit.edu/primer3/) based on equine sequences for all genes except *AMH*. The web based nucleotide BLAST program from NCBI was used to confirm that the primers were specific to each gene of interest.

Table 2.1 Primers

Genes	Accession number	Oligos sequences (5'-3')	Product size (bp)
CASP2	XM_001495660	**F:**GAGGGGTCCTCAGGCTTTTG **R:**CGGTGGGAGTACGTGCTGAA	116
CASP3	NM_001163961	**F:**GCAAACCTCAGGGAAACATTCA **R:**CTGCTCCTTTTGCTATGATCTTCTTT	125
XIAP	XM_001500906	**F:**TTTGTTTTGGGCCGGAATGT **R:**TGATCCGTGCTTCATAATCTGC	124
FST	NM_001081811	**F:**CTGGGCAGATCGATTGGATTAG **R:**GGCCTCTGCCAACCTTGAAAT	117
Tubulin	XM_001504174	**F:**GGCCAGATGCCAAGTGACAA **R:**CCGTGGGTTCCAGGTCTACA	122
GAPDH	XM_001502360	**F:**CACCCAGAAGACCGTGGATG **R:**AGGGATGACCTTGCCCACAG	118

Polymerase chain reactions (PCR) were performed using cDNA from oocytes, granulosa and cumulus cells using the Verity 96 well Thermal Cycler (AB Applied Biosystems, Inc., Carlsbad,CA). The results of the PCR reactions were visualized with ethidium bromide after electrophoresis in a 2% agarose gel. The candidate cDNA product was purified from gels using QIAquick Gel Extraction kit (Qiagen Inc, Valencia CA) and submitted for sequencing (Proteomics and Metabolomics Facility, Colorado State

University, Fort Collins CO). Identity of the cDNA amplicon was confirmed after sequencing.

AMH primers (Table 2.2) were designed based on a conserved region between human, cow, pig and mouse *AMH* sequences (see Table 2.4). Conserved regions among species were determined using a multiple alignment program (ClustalW2; http://www.ebi.ac.uk/Tools/clustalw2/index.html). A reverse transcriptase PCR reaction was performed using a pool of cDNA from granulosa and cumulus cells and the resulting product visualized by gel electrophoresis on a 2% agarose gel. The DNA product was then extracted and sequenced (Table 2.3), as described for the other genes.

Table 2.2 AMH primer

Genes	Accession number	Oligos sequences (5'-3')	Product size (bp)
AMH	NM_000479	**F:** CCGAGCGCTCCGTACTCAT **R:** CATCTTCAGCAGCAGCACCA	122

Table 2.3 *AMH* sequence obtained from PCR product

TCCCGGCCGATAGGCCACACTGCCAGGGCGCGTGCGGCTGGCCGCAGTCGGACCGC
AACCCGCGCTACGGCAACCACGTGGTGCTGCTGCTGAAGATG

Table 2.4 *AMH* sequence in the cow, pig, human and mice. Primers are highlighted.

```
Cow   GCGCTCTGCGTGAGCTGAGCGTAGACCTGCGGGCCGAGCGCTCGGTGCTCATCCCCGAGA
Pig   GTGCGCTGCGCGAACTTAGCGTGGACCTGCGCGCCGAGCGCTCGGTGCTCATCCCCGAGA
Human GCGCGCTGCGCGAGCTCAGCGTAGACCTCCGCGCCGAGCGCTCCGTACTCATCCCCGAGA
Mice  GCGCGCTGCGCGAGCTGAGTGTAGATCTGCGCGCGGAGCGTTCAGTGCTCATCCCGGAGA
      * ** ***** ** ** ** ** ** ** ** ** ***** ** ** ******** ****

Cow   CATACCAGGCCAACAACTGCCAGGGGGCCTGCGGCTGGCCTCAGTCGGACCGCAACCCGC
Pig   CATATCAAGCCAACAACTGCCAAGGCACCTGCGGCTGGCCGCAATCGGACCGCAACCCGC
Human CCTACCAGGCCAACAATTGCCAGGGCGTGTGCGGCTGGCCTCAGTCCGACCGCAACCCGC
Mice  CCTACCAAGCCAACAACTGCCAAGGCGCCTGCGCGTGGCCGCAGTCTGACCGTAATCCGC
      * ** ** ******** ***** **    ****  ***** ** ** ***** ** ****

Cow   GCTACGGCAACCACGTGGTGCTGCTGCTAAAGATGCAGGCCCGCGGCGCCACCCTGGCGC
Pig   GGTATGGCAACCACGTGGTGCTTCTGCTTAAGATGCAGGCCCGCGGCGCCGCCCTGGCCC
Human GCTACGGCAACCACGTGGTGCTGCTGCTGAAGATGCAGGCCCGTGGGGCCGCCCTGGCGC
Mice  GCTACGGGAACCACGTGGTGCTGCTGCTAAAAATGCAGGCTCGCGGGGCTGCCCTGGGCC
      * ** ** ************** ***** ** ******** ** ** **  ******  *
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Real time PCR analyses were performed in 10 μL reactions containing 2X SYBR Green Master Mix I (Roche Applied Sciences), 0.5 μM gene specific forward and reverse primer, and cDNA using the LightCycler480 PCR system (Roche Applied Sciences). The reaction conditions were as follows: 95°C for 5 minutes and then 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by a melt curve analysis. Fluorescence acquisition was performed after the extension step at the end of each cycle. Amplification efficiencies were calculated based on standard curves built for each primer and were all within 1.88 and 2.1. Relative expression of genes was calculated according to Scmittgen and Livak [135]. The threshold cycle (C_T) values for the genes of interest were normalized to the housekeeping gene specific for each cell type (*GAPDH* for granulosa and cumulus cells and *Tubulin* for oocytes), and reported as average $2^{-\Delta CT}$ values. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ (Comparative C_T method).

Hormone assays

FF samples were assayed for estradiol, androstenedione and FST. Estradiol concentrations were measured at the Colorado State Endocrine Laboratory using a radioimmunoassay (RIA) [86]. Briefly, samples were extracted using diethyl ether, resuspended in 0.1% phosphate buffer saline (PBS)-gel, then further diluted in PBS-gel to a 1:10,000 ratio. All values were determined in a single assay with a detection limit of 33.42 fg/mL, and the intra-assay coefficient of variation (CV) was <12.5%. Androstenedione concentrations in FF were quantified by solid phase RIA using a commercial kit (Siemens Medical Diagnostic Solutions, Deerfield, IL) according to the manufacturer's specifications. FF samples were diluted 1:10 in phosphate buffered saline

containing 1% gelatin (pH 7) prior to assaying 25 μL in duplicate. All values were determined in a single assay (CV=4.3%) with a detection limit of 0.16 ng/mL of undiluted FF. The assay was performed by the Animal and Range Sciences Endocrinology Laboratory at New Mexico State University. Follicles with ratios of androgens/estrogens (A/E) <1 were considered estrogen-dominant and follicles with A/E ≥1 were considered androgen-dominant.

Concentrations of FST in FF were determined with a sandwich ELISA kit (Catalog No. DNF00; R & D Systems, Inc., Minneapolis, MN). The kit was developed for use with human serum and FF, and was adapted and validated for use with equine FF by Ginther and collaborators [56]. According to the manufacturer, there is no significant cross-reactivity of the assay with recombinant human activin-A, inhibin-A and -B and other cytokines. FF FST concentrations were determined in all samples collected from preovulatory follicles after induction of maturation, from both groups (young and old). In addition to the FF samples collected concomitantly with the oocytes and follicular cells, additional FF samples were collected during the breeding season of 2009, in order to describe the pattern of FST in the dominant follicle after deviation. Therefore samples from both groups were collected from different size follicles: post-deviation (25 mm); mid-estrus (30 mm); preovulatory follicles (35 mm, 30-36 h post induction of maturation). FF samples were diluted to a 1:60 ratio, using the diluent provided with the kit, and analyzed in two assays. The intra-assay CV for quality control samples of the first assay was 12.6%, and the sensitivity was 0.142 ng/mL as determined by 2 standard deviations above the mean optical density of the zero standard. For the second assay, the

intra-assay CV and sensitivity were 22.6% and 0.08 ng/mL respectively. The inter-assay CV was 17.4%.

For the AMH ELISA assay validation procedure see Apendix I.

Statistical analyses

Statistical differences in the normalized relative expression data, resulting from real time PCR analyses, were assessed at $P<0.05$ using a non-paired, two-tailed Student t-test. Androstenedione and estradiol FF concentrations from young and old mares were analyzed using a non-paired, two-tailed Student t-test, differences were assessed at $P<0.05$. FST FF data was log-transformed to correct for non-homogeneous variance, and a two-way analysis of variance (ANOVA) was performed using SAS 9.2 (Statistical Analysis System, Cary, NC) in order to assess differences between age groups and among time-points.

CHAPTER IV: RESULTS

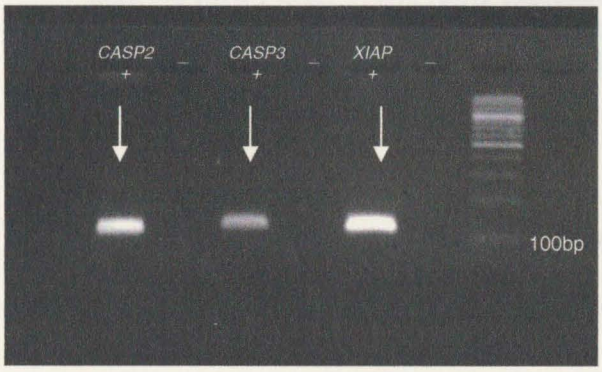
A total of 52 follicular aspirations were performed and 24 oocytes were recovered. Mean follicular diameter was 38.8 ± 2.8 mm at the day of aspiration. Only oocytes that were considered to be mature, based on the presence of light sheets of granulosa cells with soft borders and an expanded cumulus, were included in the experiment. Due to technical difficulties, not all of the different follicular components (granulosa cells Y=10, O=12; cumulus cells Y=10, O=12; oocyte Y=10, O=14; follicular fluid Y=8, O=8) were collected from each individual aspiration. Furthermore, due to variability between mares, we were unable to obtain gene expression data for all samples collected. We obtained housekeeping gene expression for all samples, except for two oocytes, that were excluded from the analysis (Table 3.1).

Table 3.1 Number of samples per gene per cell type. For the samples collected from Young (Y) and Old (O) mares, total samples for oocytes (Oc), granulosa cells (Gc) and cumulus cells (Cc) are provided (Samples). Gene expression data for each gene, within Y vs. O, for each cell type is provided as not all samples returned measurable expression values for all genes.

	Samples		*GAPDH*		*Tubulin*		*AMH*		*FST*		*XIAP*		*CASP2*		*CASP3*	
	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O
Oc	10	14	10	12	10	12	_	_	4	3	10	7	10	6	10	10
Gc	10	12	10	12	10	12	9	8	_	_	10	9	_	_	9	11
Cc	10	12	10	12	10	12	9	6	_	_	9	8	_	_	9	8

Qualitative expression of *XIAP*, *CASP3*, *CASP2* (FIG. 3.1 A) and *FST* (FIG. 3.1 B) in equine oocytes was demonstrated by visualization of the PCR reaction products in 2% agarose gels after electrophoresis. Qualitative expression of *XIAP*, *CASP3*, *CASP2* and *AMH* in equine granulosa (Fig. 3.2) and cumulus cells was also demonstrated (Fig. 3.3).

A



B

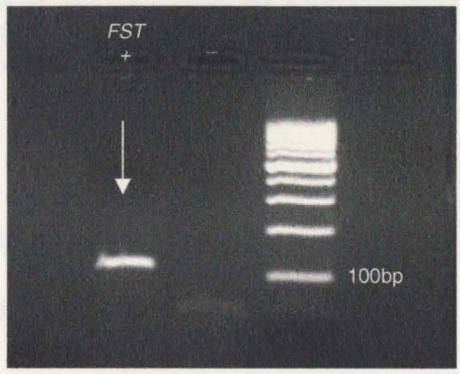


Figure 3.1 *XIAP*, *CASP3*, *CASP2* and *FST* expression in equine oocytes. RT-PCR reactions were performed using a cDNA pool of oocytes, negative controls (-) for each gene were constructed with no addition of cDNA. Ladder is depicted to the right of samples with the 100 bp band marked.

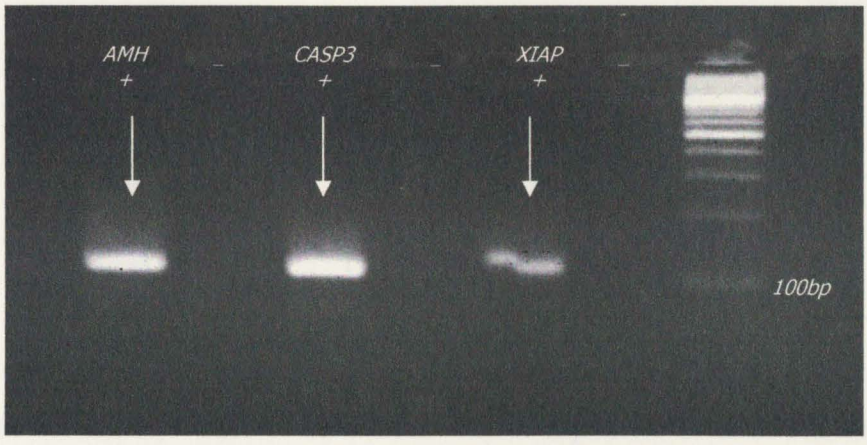


Figure 3.2 *XIAP*, *CASP3*, and *AMH* expression in equine granulosa cells. RT-PCR reactions were performed using a cDNA pool of granulosa cells, negative controls (-) for each gene were constructed with no addition of cDNA. Ladder is depicted to the right of samples with the 100 bp band marked.

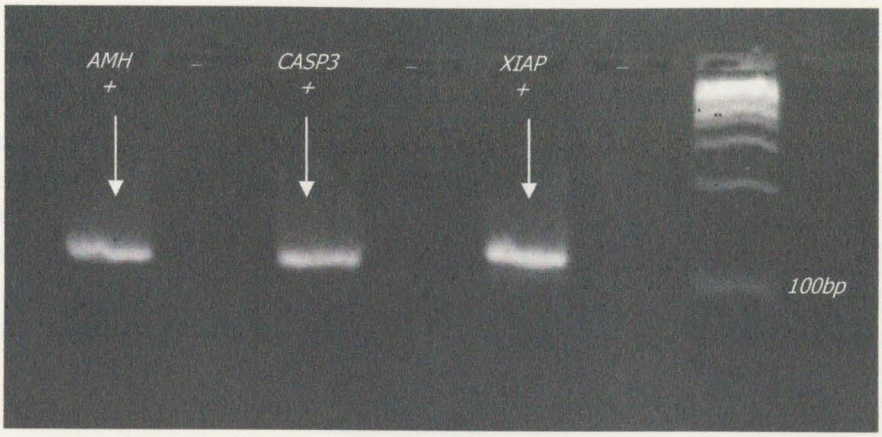


Figure 3.3 *XIAP*, *CASP3*, and *AMH* expression in equine cumulus cells. RT-PCR reactions were performed using a cDNA pool of cumulus cells, negative controls (-) for each gene were constructed with no addition of cDNA. Ladder is depicted to the right of samples with the 100 bp band marked.

Based on real time analysis, there was no difference in the expression levels of *CASP2* (P=0.251; t-test) (Fig. 3.4A) and *CASP3* (P=0.5; t-test) (Fig. 3.4B) between oocytes from young and old mares. In addition there was no significant difference (P=0.1; t-test) in the expression levels of *CASP3* in granulosa cells (Fig. 3.5B) and cumulus cells (P=0.7; t-test) (Fig. 3.5A) between young and old mares.

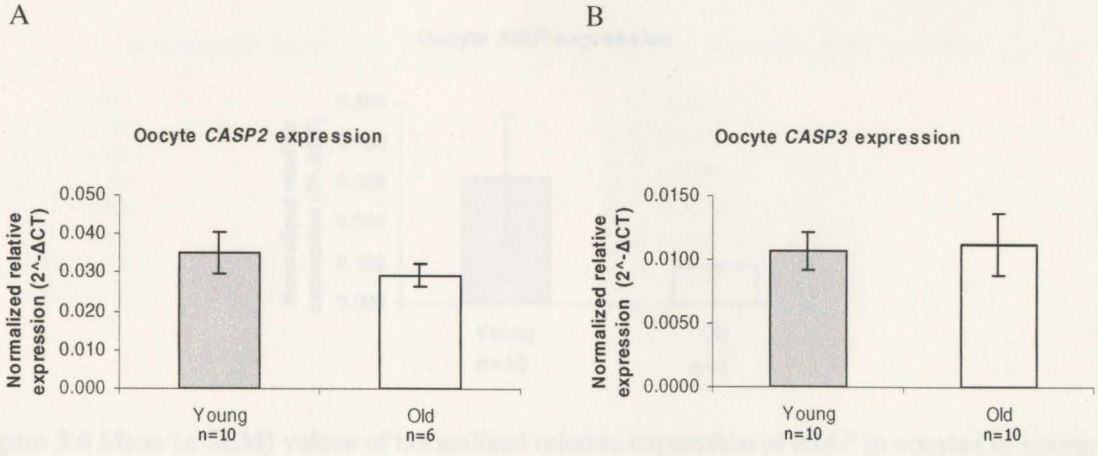


Figure 3.4 Mean (±SEM) values of normalized relative expression of *CASP2* (P=0.251; t-test) and *CASP3* (P=0.5; t-test) from oocytes of young vs. old mares.

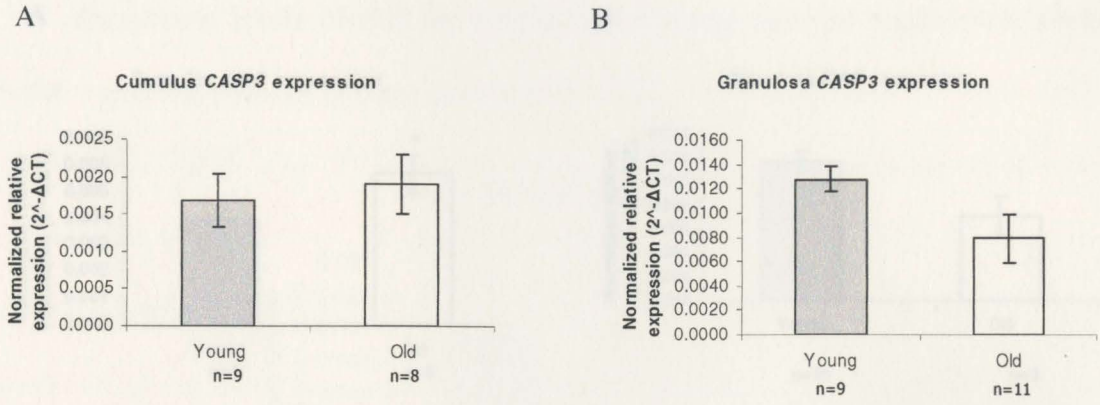


Figure 3.5 Mean (± SEM) values of normalized relative expression of *CASP3* in granulosa (P=0.1; t-test) and cumulus cells (P=0.7; t-test) from young vs. old mares. There was no statistical difference in expression levels between age groups.

Quantitative expression levels of *XIAP* were 3.3 fold higher in oocytes from young mares than oocytes from old mares (P=0.045; t-test) (Fig. 3.6). In granulosa cells, the difference in *XIAP* normalized relative expression between young and old mares approached significance (P=0.06; t-test), with a tendency for higher expression in young mares than in old mares (Fig. 3.7B). In cumulus cells *XIAP* expression levels were 1.46 fold higher in old mares as compared to young mares (P=0.046; t-test) (Fig. 3.7A).

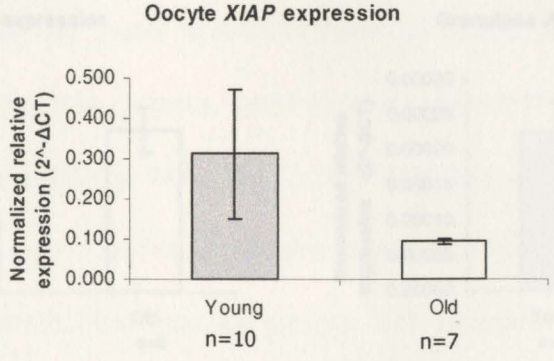


Figure 3.6 Mean (± SEM) values of normalized relative expression of *XIAP* in oocytes of young vs. old mares (P=0.045; t-test).

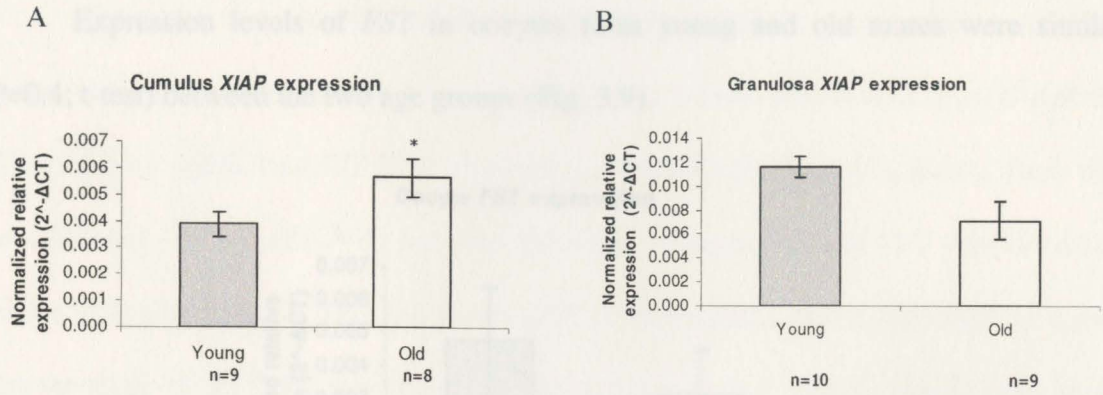


Figure 3.7 Mean (± SEM) values of normalized relative expression of *XIAP* in granulosa (P=0.06; t-test) and cumulus cells (P=0.046; t-test) from young vs. old mares.

There was no significant difference (P=0.2; t-test) in the expression levels of *AMH* in granulosa cells between young and old mares (Fig. 3.8B). In cumulus cells, the difference in *AMH* expression between young and old mares approached significance (P=0.078, t-test), with a tendency for higher expression in cells from old vs. young mares (Fig. 3.8A).

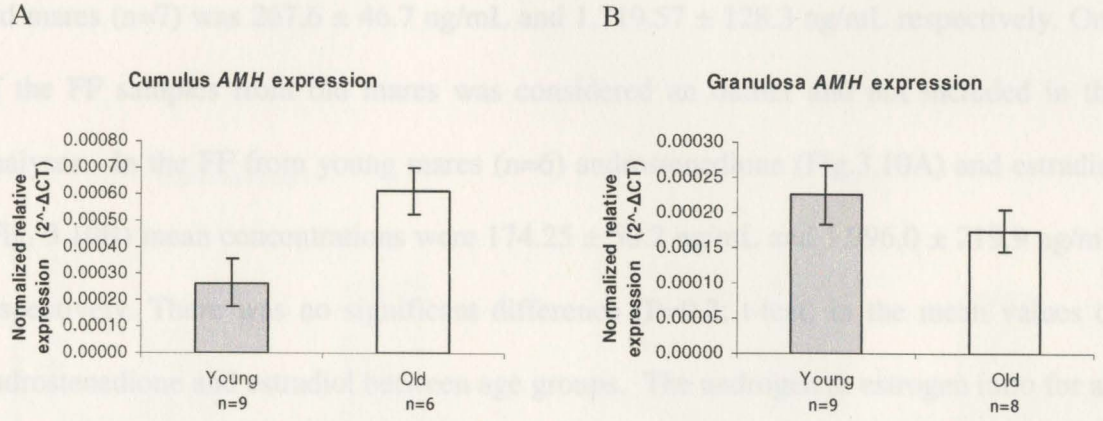


Figure 3.8 Mean (± SEM) values of normalized relative expression of *AMH* in granulosa (P=0.2; t-test) and cumulus cells (P=0.078, t-test) from young vs. old mares.

Expression levels of *FST* in oocytes from young and old mares were similar (P=0.4; t-test) between the two age groups (Fig. 3.9).

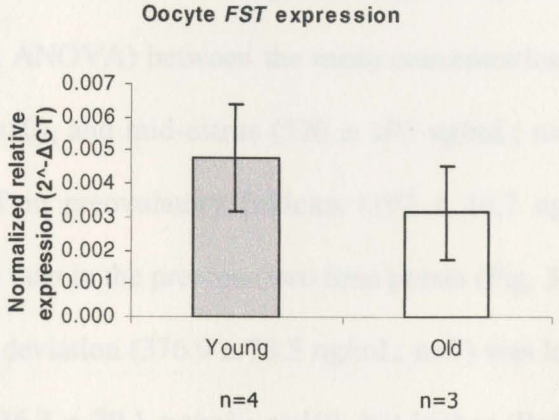


Figure 3.9 Mean (± SEM) values of normalized relative expression of *FST* in oocytes (P=0.4; t-test) from young vs. old mares.

The mean follicular fluid concentrations of androstenedione and estradiol were 227.59 ± 35.37 ng/mL and 1,847.15 ± 123.75 ng/mL respectively. The mean concentration of androstenedione (Fig. 3.10A) and estradiol (Fig. 3.10B) in the FF from old mares (n=7) was 267.6 ± 46.7 ng/mL and 1,719.57 ± 128.3 ng/mL respectively. One of the FF samples from old mares was considered an outlier and not included in the analyses. In the FF from young mares (n=6) androstenedione (Fig.3.10A) and estradiol (Fig. 3.10B) mean concentrations were 174.25 ± 50.2 ng/mL and 1,996.0 ± 219.9 ng/mL respectively. There was no significant difference (P=0.2; t-test) in the mean values of androstenedione and estradiol between age groups. The androgen to estrogen ratio for all samples was less than one, therefore all follicles were considered estrogen-dominant (not undergoing atresia). The difference between the androgen to estrogen ratio between young and old mares approached significance (P=0.07, t-test), with a tendency of a higher androgen to estrogen ratio in FF from young mares vs. old mares.

The mean concentration of FST in the largest follicle post-deviation (25mm), during mid-estrus (30mm) and prior to ovulation (35mm) was 467.21 ± 41.0 ng/mL, 578.15 ± 64.8 ng/mL and 175.77 ± 14.4 ng/mL respectively. In young mares, there was no difference (P=0.5; ANOVA) between the mean concentrations of FST post-deviation (530 ±38.4ng/mL; n=10), and mid-estrus (520 ± 103 ng/mL; n=10), although the mean concentration of FST in preovulatory follicles (197 ± 16.7 ng/mL; n=18) was lower (P<0.0002; ANOVA) than in the previous two time points (Fig. 3.11). In old mares, mean FST concentration at deviation (376.9 ± 73.5 ng/mL; n=7) was lower (P=0.04; ANOVA) than at mid-estrus (636.3 ± 79.1 ng/mL; n=10), but higher (P=0.02; ANOVA) than the mean concentration in follicular fluid of preovulatory follicles (153.3 ± 22.7 ng/mL; n=18) (Fig. 3.11). The mean FST concentration in follicular fluid of preovulatory follicles of old mares was lower (P<0.0001; ANOVA) than the two previous time points (Fig 3.11). FST concentrations in preovulatory follicles of young mares were higher (P=0.02; ANOVA) than in FF of old mares.

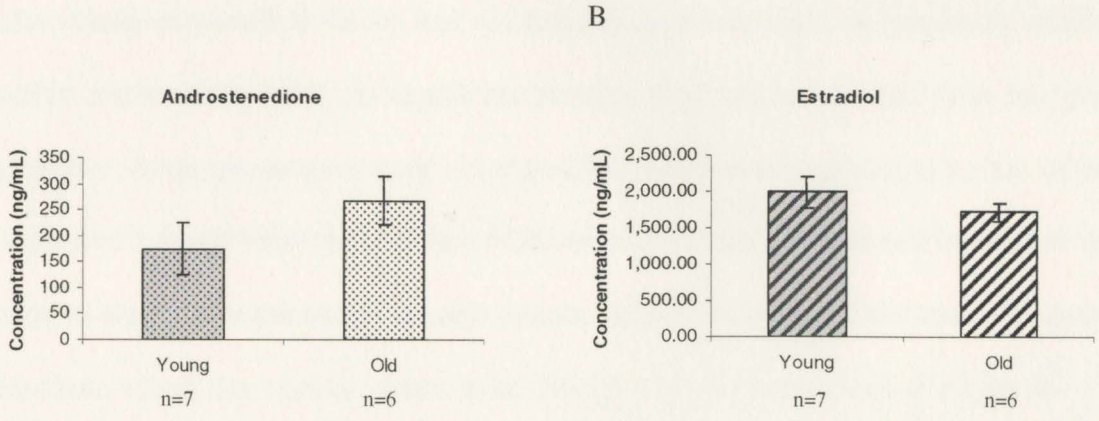


Figure 3.10 Mean (± SEM) follicular fluid concentrations of androstenedione and estradiol (P=0.2; t-test) in young vs. old mares.

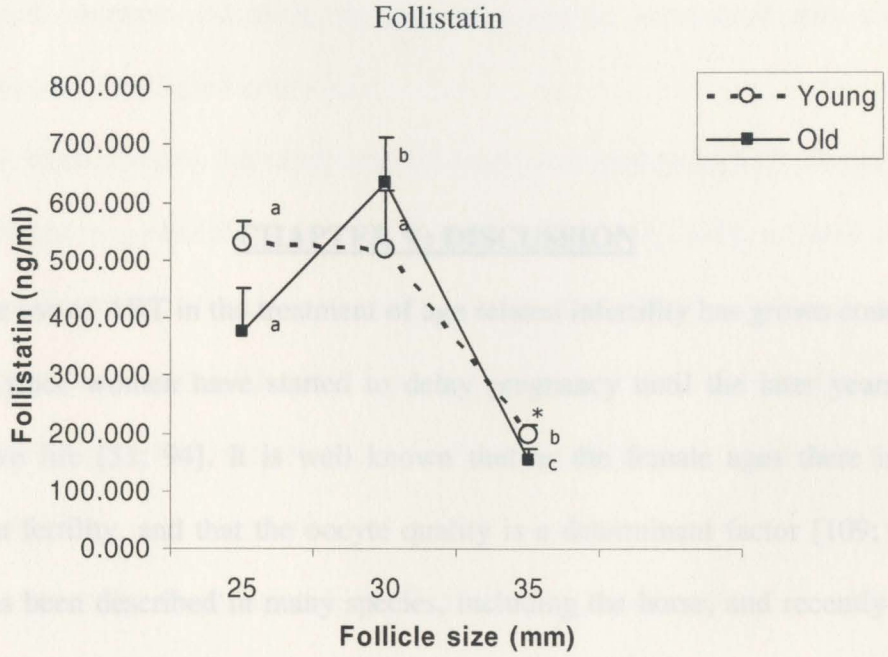


Figure 3.12 Follistatin mean (± SEM) concentrations from FF of young (Y) vs. old (O) mares collected at different time points [post-deviation (25 mm), mid-estrus (30 mm), preovulatory follicles (35 mm)]. Number of samples for each age group, per time-point, were as follows: 25mm (Y) n=10, (O) n=7; 30 mm (Y) n=10, (O) n=10; 35 mm (Y) n=18, (O) n=18. Within a group, means with no common letters (a,b,c) are different ($P<0.05$; ANOVA). * Denotes statistical difference between age groups within a time point ($P=0.02$; ANOVA).

CHAPTER V: DISCUSSION

The use of ART in the treatment of age related infertility has grown considerably, especially since women have started to delay pregnancy until the later years of their reproductive life [51; 94]. It is well known that as the female ages there is a serial decrease in fertility, and that the oocyte quality is a determinant factor [109; 18]. This process has been described in many species, including the horse, and recently the mare has been described as a suitable model to study age related fertility in women [20].

Starting from the principle that oocytes from young mares are more competent than oocytes from old mares, we wanted to assess the involvement of apoptosis of follicular cells and/or of the oocyte in the process of fertility loss that occurs in old mares. We also investigated levels of FST and AMH in different follicular cell types and follicular fluid. By doing so, we wanted to identify possible markers that are differentially expressed in young and old females, and that could be potentially used as fertility markers for ART. A significant problem that we encountered was the great variability within the samples from old mares, but this is not surprising since not all old mares have reduced fertility. Real time PCR results from the different cell types from old mares in most cases presented variable results, with some samples not even presenting detectable values for certain genes, even though all samples presented results for the housekeeping genes. Therefore, some of the statistical analyses were performed on

relative small numbers. As such, the results should be considered with caution, we believe they are valuable information.

The identification of fertility markers is of great interest to ART. Recently, AMH was considered a potential marker for oocyte quality [44; 141], as well as a good indicator of the onset of menopause [80], and an individual's ability to respond to superovulation treatments [43]. AMH is a member of the TGF-β superfamily and besides its role in fetal sex differentiation is involved in the regulation of follicular growth and development [91].

In our study, we compared *AMH* mRNA expression levels in granulosa and cumulus cells between young and old mares, and we also sought to validate the use of a human AMH ELISA assay (DSL-10-14400; Diagnostic Systems laboratories INC, Webster, TX) to quantify AMH concentrations in FF and serum of mares (see Appendix I). We were able to demonstrate *AMH* expression in equine granulosa and cumulus cells, confirming previous reports [4]. However, real time PCR results showed no difference in the expression levels between granulosa cells from young and old mares.

In cumulus cells, mRNA levels of *AMH* were not significantly different between young and old mares, but there was a tendency for *AMH* to be more highly expressed in cells from old mares. As the follicle grows, *AMH* mRNA expression increases in human cumulus cells and decreases in granulosa mural cells [158]. Higher expression of *AMH* in cumulus cells of old mares was unexpected as such a pattern has not been described. In humans, reproductive aging is marked by hormonal dysregulation that includes alterations in GnRH pulses, and FSH patterns [150; 162]. In addition to the alterations in hormonal levels, dysregulation in the pattern of expression of several genes in ovarian

follicular cells also occurs [70]. The aging mare presents the same pattern of alterations in normal reproductive physiology [20], and dysregulation at the level of gene expression in follicular cells could explain the tendency to have higher levels of *AMH* in cumulus cells of old mares. In addition, we only obtained data from cumulus cell samples from 6 old mares and 9 young mares, consequently a larger sample size is warranted.

Our attempts to validate the human AMH ELISA assay for use with equine samples were not successful. Nevertheless, the development of a quantitative assay to measure AMH in serum and FF of mares could be a great advantage to equine reproduction. In addition to being a possible marker of oocyte quality, there are many benefits to being able to quantitatively measure AMH in the horse. For instance, Ball and collaborators demonstrated the presence of active AMH in the serum of mares, and showed that mares with granulosa cell tumors had higher immunolabeling of AMH in granulosa cells [4]. Therefore, they proposed AMH as a possible marker for granulosa cell tumors in the mare. Moreover, due to the relationship between serum AMH and antral follicle count in cattle [73] and humans [27; 149], and the fact that AMH has been considered a good indicator of ovarian aging [80], AMH serum levels could also be a useful tool in the assessment of the reproductive status of mares.

In addition to AMH, FST may serve as a potential marker for oocyte quality. FST is an activin binding protein and exerts its actions mainly by regulating bio-availability of activin, but it also binds inhibin and BMPs with lower affinity [63]. FST, activin and BMPs have been suggested to be local regulators of follicular development [160]. Recent studies comparing oocytes from pre-pubertal calves and adult cows, and studies utilizing RNA interference techniques to decrease *FST* mRNA levels in oocytes, reported that

oocytes containing higher levels of *FST* mRNA are more competent and proposed a role for *FST* in early embryonic development in the bovine [114; 93]. In our experiments, we were able to demonstrate qualitative *FST* expression in equine oocytes for the first time. Unfortunately, we only obtained real time PCR results from a small number of oocytes (4 young and 3 old) and were not able to show a statistical difference in *FST* mRNA levels in oocytes from young and old mares. Therefore, further studies using larger sample numbers are warranted to elucidate the relationship between *FST* mRNA levels and oocyte quality.

FST is also expressed in granulosa cells and results in measurable concentrations in follicular fluid. *FST* mRNA expression in granulosa cells and FST follicular fluid concentrations increase as the follicle grows in humans [136] and cattle [137]. In the horse, Ginther and collaborators reported a mean FST concentration in the follicular fluid of mares, in the range of 350 ng/mL and 180 ng/mL for the biggest and second biggest follicle respectively, at the time of deviation [56]. No further work has been done investigating FST concentrations during follicular growth in the horse.

While we were not able to detect any differences in mRNA levels of *FST* in oocytes from young and old mares, when we examined protein levels of FST in FF, collected at different follicular stages (post-deviation, mid-estrus, preovulatory), we observed two phenomena worth noting. First, FST concentration levels were higher in young vs. old mares in post-deviation (25 mm) and preovulatory (35 mm) follicles, but not in follicles during mid-estrus (30 mm). Studies based on FST FF concentrations in women undergoing IVF treatments have suggested that follicles containing FF with higher levels of FST resulted in a higher number of mature oocytes [50]. Therefore FST

levels in equine follicular fluid could be a potential marker of oocyte quality. Nonetheless, further studies investigating the relationship between FST FF concentrations, cleavage rates, blastocyst formation and pregnancy outcome after *in vitro* fertilization procedures are still necessary in the horse.

In addition to the difference in FST levels between young and old mares during follicular development, we also observed that FST FF concentrations decreased in preovulatory follicles of both groups. In young mares, FST concentrations remained constant in 30 mm follicles when compared to 25 mm follicles, but were significantly lower in preovulatory follicles. In old mares, FST levels significantly increased in 30 mm follicles when compared to 25 mm follicles, and subsequently decreased in preovulatory follicles. This fluctuation in FST levels during follicular growth in the old mare exemplifies again the dysregulation in normal physiology.

The decrease in FST levels in equine preovulatory follicles was unexpected, since this pattern of FST in equine FF that we describe differs from that reported in humans and cattle [136; 137]. However, it seems to be similar to the pattern reported in the ewe [161], the sow [95] and the rat [85], where *FST* mRNA levels in granulosa cells decrease as the follicle grows [146; 161]. In sheep, *FST* mRNA expression decreased markedly after the LH surge [161]. In our experiment, samples from preovulatory follicles were collected 30-36 h post induction of maturation using an hCG and deslorelin combination to mimic and induce the LH surge. Therefore, in the mare as in the ewe, the LH surge could be signaling to granulosa cells and decreasing *FST* expression prior to ovulation and luteinization of the follicle. The reason for this down-regulation in these species is

not known, the mechanism involved in this process is not clear, and further studies are required.

Because of the importance of follicular cells to oocyte development and the controversy regarding apoptosis in follicular cells relative to oocyte quality, we investigated the expression of pro-apoptotic and pro-survival genes in follicular and germ cells. We quantified expression of *XIAP* and *CASP3* in granulosa cells, cumulus cells, and oocytes from young and old mares. We also analyzed expression of *CASP2* in oocytes from both age groups. Moreover, to assess if the follicles that were sampled were undergoing atresia we determined the androgen to estrogen ratio of the follicles. All follicles analyzed had an androgen to estrogen ration of less than one and therefore were not considered atretic. Real time PCR results showed no difference in *CASP3* expression levels in granulosa and cumulus cells between the two age groups. In addition, there was no difference in *CASP2* and *CASP3* mRNA expression in oocytes from young and old mares.

Based on these results we do not believe that mRNA expression levels of caspase-3 in follicular cells, and caspases 3 and 2 in the oocyte, directly affect fertility in the old mare. However, further studies measuring the levels of active caspases in these cell types, and utilizing assays specific for the detection of apoptosis at different follicular stages, in association with *in vitro* fertilization procedures, are necessary to completely elucidate the involvement of apoptosis in oocyte quality and fertility loss in the old mare. In addition, it is important to note that during sample collections we evaluated follicular development as well as oocyte and follicular cells morphology to ensure that the samples were being collected from mature, healthy follicles from both age groups. The strict

selection during sampling could also explain the similar results obtained from the analyses of pro-apoptotic genes in samples from young and old mares. Furthermore, some of the studies reporting a correlation between granulosa cell apoptosis and oocyte maturity and quality were performed using ovaries obtained from abattoirs, what could also explain the different results reported by them.

The data presented here also demonstrates that *XIAP* mRNA levels are expressed 3.3 fold higher in oocytes from young when compared to old mares. It has been shown that higher mRNA levels of *XIAP* provide cells with protection against deleterious effects, hence preventing apoptosis [2]. A recent study using bovine embryos demonstrated that leptin treatment increased the content of *XIAP* mRNA in embryos and resulted in better development rates [9]. Besides, another member of the IAP family, *survivin*, might be playing a role as a maternal effect gene in mice oocytes, positively influencing embryo development [132]. Furthermore, *survivin* levels were lower in oocytes from aged mice [132]. Therefore maternal derived *XIAP* could also be playing an important role during early embryonic development in the horse. In that case, accumulation of *XIAP* mRNA during follicular growth would be crucial, and the amount of *XIAP* stored in the oocyte until ovulation could influence its ability to be fertilized, cleave, and develop into a blastocyst. Further studies evaluating *XIAP* mRNA levels in oocytes at different follicular stages and during early embryonic development are required to confirm this hypothesis.

In addition, there was a tendency for *XIAP* to be more highly expressed in granulosa cells of young mares. This result suggests that granulosa cells from young mares are more resistant to apoptosis, and maybe provide a more suitable environment

for the development of the oocyte. In contrast, the levels of *XIAP* mRNA in cumulus cells were 1.46 fold higher in old when compared to young mares. This result is not in accordance with our hypothesis, and could reflect dysregulation of key physiological events in follicles of old mares. However, arguably this result supports the notion that apoptosis of follicular cells is a good indicator of oocyte maturity and developmental potential. If correct, then cumulus cells from old mares could have lower rates of apoptosis, indicating lower oocyte competency.

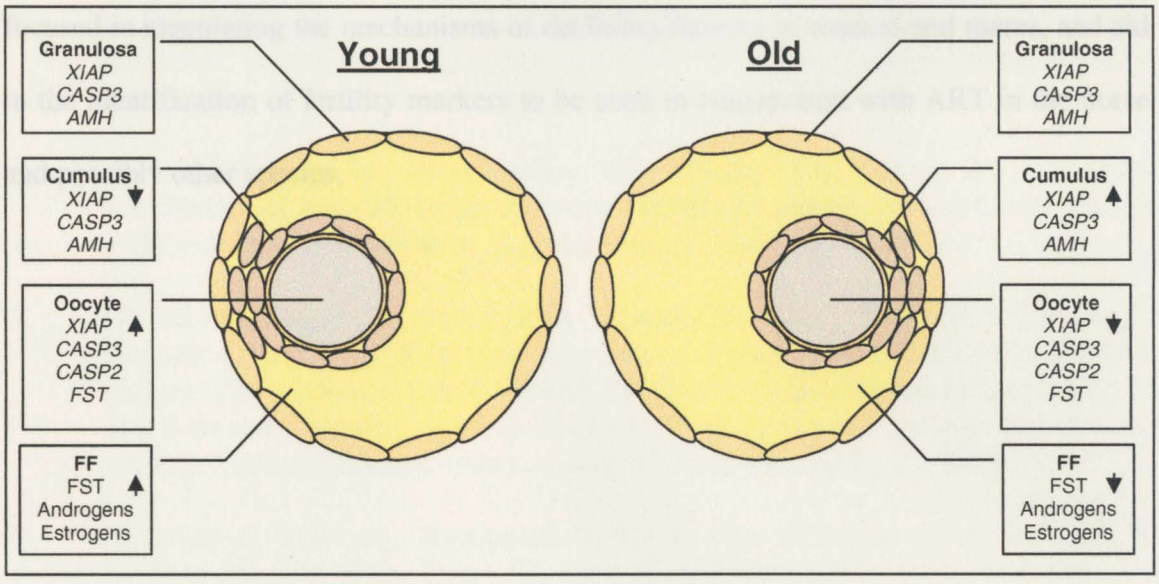


Figure 5.1 Preovulatory follicles from young and old mares. Summary of genes analyzed in each cell type and hormonal concentrations in FF of young and old mares. Arrows in the legends denote statistical differences.

In conclusion, we believe that our data suggest that FST follicular fluid levels can be a non-invasive marker to assess oocyte quality in the horse, and that FST levels decrease in preovulatory follicles of the horse. In addition, expression levels of caspase-3 in follicular cells, and caspases 3 and 2 in the oocyte, does not seem to be involved in the

mechanism of fertility loss in the old mare. Finally, *XIAP* mRNA levels may be important for oocyte quality in the horse.

The use of ART in the equine industry is increasing. New advances, are, however, slow in coming. The reasons for that are multi-fold and include the costs of performing research in the horse and obtaining high quality samples. Nevertheless, we need to develop new techniques and improve the current ones, for both the inherent value to the horse industry as well as fully developing the mare as a research model for the human. We believe that the data presented here will help direct the development of research focused in elucidating the mechanisms of declining fertility in women and mares, and aid in the identification of fertility markers to be used in conjunction with ART in the horse and possibly other species.

CHAPTER VI: REFERENCES

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APPENDIX I: AMH ELISA ASSAY VALIDATION

We attempted to determine AMH concentrations in equine serum and FF using a human AMH sandwich ELISA kit (DSL-10-14400; Diagnostic Systems Laboratory, Webster, TX). The kit was developed for use with human serum and FF, therefore it was necessary to validate its use with equine samples. Follicular fluid samples from young (n=10) and old (n=10) mares were collected from pre-ovulatory follicles through transvaginal follicular aspirations as previously described (see Chapter III: Materials and Methods). After collection FF samples were centrifuged at 5ºC to eliminate debris and then stored at -80ºC. The standards (14-0 ng/mL) and quality controls (8 and 2 ng/mL) used were provided by the manufacturer with the kit.

Different amounts (1, 20, 60, 100 µL) of a pool of equine FF in a total volume of 100 µL of assay diluent resulted in a displacement curve that was similar to the standard curve (Fig. A.1). In addition, a sample of pool FF was spiked with different amounts (1.75, 3.5 and 7 ng/µL) of exogenous human AMH, but we failed to demonstrate a proportional increase in the absorbance values (Fig. A.2). Furthermore, when we assayed different FF samples using 2 different dilutions (1:20 and 1:10) we failed to detect any difference in absorbance values between the dilutions. Therefore we concluded that this ELISA assay it is not suitable to investigate AMH concentrations in equine follicular fluid.

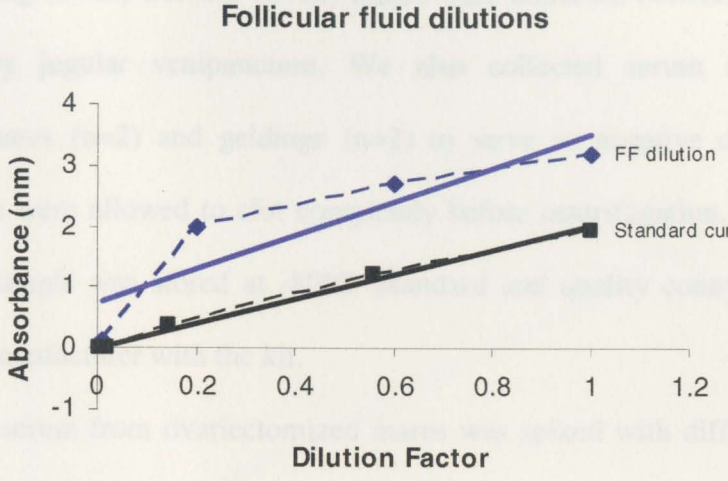

Figure A.1- Follicular fluid serial dilutions. The following amounts of follicular fluid, 1, 20, 60, 100 µL, were added to assay diluent to reach a total volume of 100 µL.

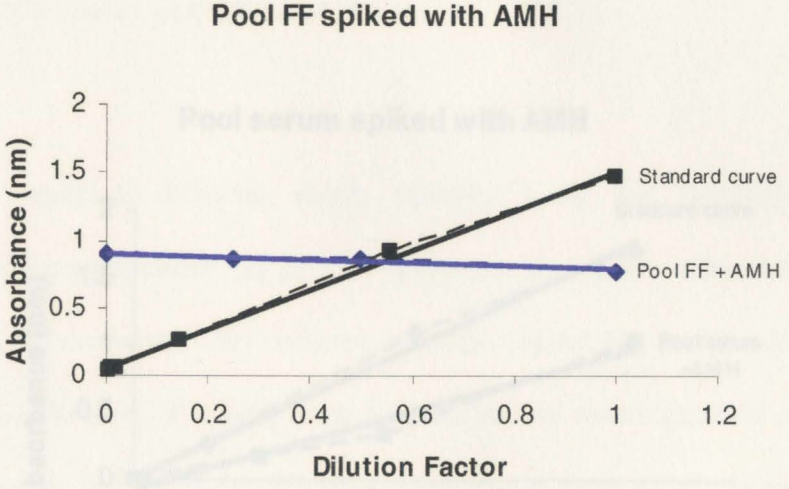

Figure A.2- A sample of pool follicular fluid was spiked with different amounts (1.75, 3.5, 7ng/mL) of exogenous human AMH.

Next, we sought to validate the assay for use with equine serum samples. Blood samples from young (n=10) and old (n=10) mares were collected between day 5 and 7 post ovulation by jugular venipuncture. We also collected serum samples from ovariectomized mares (n=2) and geldings (n=2) to serve as negative controls. After collection samples were allowed to clot completely before centrifugation, after which 1 mL of cell free sample was stored at -80°C. Standard and quality controls used were provided by the manufacturer with the kit.

A pool of serum from ovariectomized mares was spiked with different amounts (1.75, 3.5 and 7 ng/μL) of exogenous human AMH. The serial addition of exogenous AMH resulted in a serial increase in the absorbance values, resulting in a displacement curve close to the standard curve (Fig. A.3). The negative controls obtained absorbance readings similar to those obtained from the 0 standard.

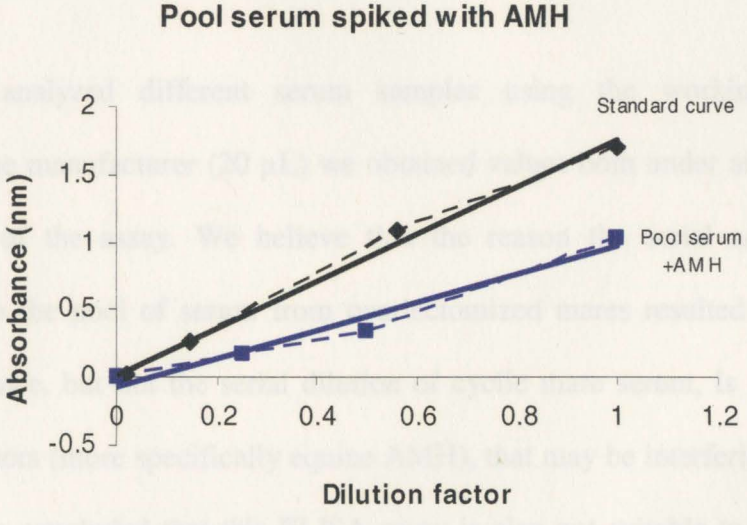

Figure A.3- A sample of pool serum from ovariectomized mares was spiked with different amounts (1.75, 3.5, 7ng/mL) of exogenous human AMH.

Although, when we used different dilutions (10, 20 and 40 μL) of a serum sample from a cyclic mare in a total volume of 100 μL of assay diluent, we did not observe any difference in the absorbance values (Fig. A.4).

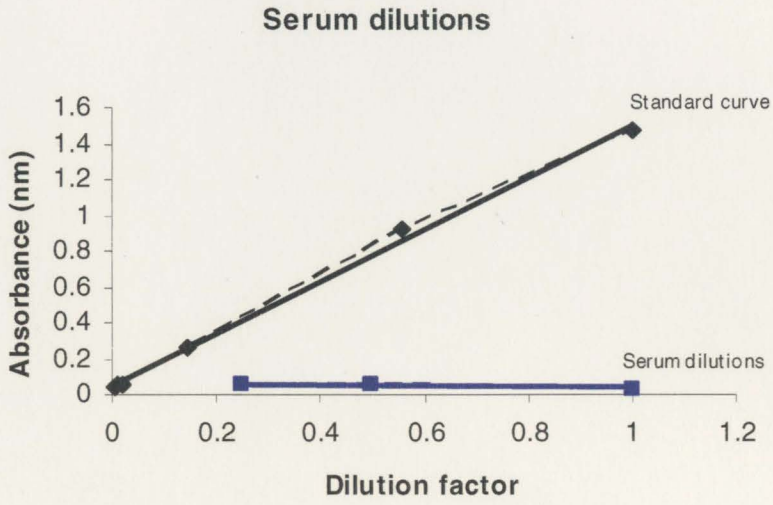

Figure A.4- Serum serial dilutions. The following amounts of serum, 10, 20 and 40 μL, were added to assay diluent to reach a total volume of 100 μL.

When we analyzed different serum samples using the working dilution recommended by the manufacturer (20 μL) we obtained values both under and over the limit of detection of the assay. We believe that the reason the serial additions of exogenous AMH to the pool of serum from ovariectomized mares resulted in a serial increase in absorbance, but not the serial dilution of cyclic mare serum, is the lack of ovarian derived factors (more specifically equine AMH), that may be interfering with the assay. Therefore we concluded that this ELISA assay is also not suitable to investigate AMH concentrations in equine serum samples.