CHANGES IN FOLLICULAR FLUID STEROIDS, INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBP) AND IGFBP PROTEO-LYTIC ACTIVITY DURING EQUINE FOLLICULAR DEVELOPMENT

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TABLE OF CONTENTS

Chapter

LIST OF TABLES

LIST OF FIGURES

Figure Page

NOMENCLATURE

A_4	androstenedione
ADU	arbitrary denitometric units
BDP	breakdown product
CAA	cyclohexylacetyl-phe-arg-ser-val-gln amide
cAMP	cyclicAMP
CL	corpus luteum
cm	centimeter
cpm	counts per minute
CV	coefficient of variance
E2	estradiol
EGF	epidermal growth factor
FF	follicular fluid
FSH	follicle stimulating hormone
GH	growth hormone
h	hour
hCG	human chorionic gonadotropin
IGFBP	insulin-like growth factor binding protein
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
kDa	kilodalton

LH	luteinizing hormone
min	minutes
ml	milliliter
mm	millimeter
mM	milliMolar
mRNA	messenger RNA
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
P_4	progesterone
pg	picogram
PGE_2	prostaglandin-E_2
PGF_1	prostaglandin-F_1
$PGF_{2\alpha}$	prostaglandin-$F_{2\alpha}$
RIA	radioimmunoassay
s	second
SE	standard error
SEM	standard error means
TBS	tris-buffered saline

CHAPTER I

INTRODUCTION

The mare is a seasonal breeder, with the typical ovulatory period for mares in the Northern Hemisphere being between mid-April and mid-September. The estrous cycle is approximately 22 days in length, while estrus usually lasts 3 to 7 days. Most often, one large graafian follicle is selected to continue its growth becoming a dominant follicle, while the remaining large antral follicles undergo atresia. The ovulatory follicle in the mare can range from 35 to 55 mm in diameter, with ovulation generally occurring 24 to 48 h before the end of estrus. In cultured granulosa and thecal cells, IGF-I has dramatic stimulatory effects on steroidogenesis and mitogenesis (Adashi et al., 1985; Cara and Rosenfield, 1988; Hammond et al., 1988b). As follicle size increases, concentrations of IGF-I in follicular fluid increase (Hammond et al., 1988a; Spicer et al., 1988). Spicer et al., (1991) observed a significant correlation between follicular diameter and IGF-I concentrations in mares. Dramatic changes in steroidogenesis and levels of in IGFBP occur during the final growth of ovulatory follicles as well as during follicular atresia in the mare (Gerard and Monget, 1998). These changes in IGFs and IGFBPs could be important in follicular development, ovulation and (or) atresia (Spicer and Echternkamp, 1995). There may be variation in species-specific mechanisms related to IGF or IGFBP that regulates follicular development. More information is needed to help understand the physiological modulators that affect follicular development and maturation in the mare.

Therefore, the present study evaluated changes in FF steroid and IGFBP levels as well as IGFBP proteolysis during follicular development in the mare.

CHAPTER II

REVIEW OF LITERATURE

MARE ESTROUS CYCLE

The mare has a seasonally polyestrous type of estrous cycle, which means that she is receptive to the stallion and ovulates during certain times of the year (Ginther, 1974). Follicular activity is suppressed in the months of short day length but as day length increases, the number and size of follicles will increase (Ginther, 1979). The mare's seasonal changes are divided into two categories, cyclic and anestrus, with a spring and fall transition period between them. Typical ovulatory period for mares in the Northern hemisphere is between mid-April and mid-September. The mare will show behavioral signs of sexual receptivity; such as interest in the stallion, winking of her vulva and frequent urination; and develop follicles that will ovulate. However, there are some mares that will exhibit signs of sexual receptivity throughout the year (Ginther, 1979). The estrous cycle, which is approximately 22 days in length, is divided into two physiological stages, estrus and diestrus. Estrus, which usually lasts 3 to 7 days, is when a mare expresses signs of standing heat and final follicular maturation and ovulation occur. Serum progestin concentrations need to be less than 1 ng/ml before estrus will occur in mares (Stabenfeldt et al., 1972). Diestrus, which typically lasts for 15 to 19 days, is the time when the mare is un-receptive to the stallion. Ovulation will generally

occur 24 to 48 h prior to the end of estrus. On the 5th day of estrus, ovulation occurs, and within 24 h there is an increase in plasma progestins concentration (Stabenfeldt et al., 1972).

Ovarian Structures

In mares, the ovary is much larger on a body weight basis than other farm species (Ginther, 1992). The ovary is kidney shaped and has what is called an ovulation fossa. The only area that ovulation can occur is at the ovulation fossa. What makes the mare unique is that the medulla and cortex are inversed from that of other species. The cortex is on the inside and the thick, connective layer of medulla covers the surface of the ovary with the exception of the ovulation fossa. The ovulatory follicle in the mare can range from 35 to 55 mm in size; a possible reason for this large size is that the follicle must grow towards the ovulation fossa to ovulate. The average diameter of the preovulatory follicle on the day before ovulation was 44 mm (Ginther et al., 1989). The right ovary is often attached 15 cm behind the right kidney, and the left ovary is usually further back than the right (Sisson and Grossman, 1953). Ovarian weight during the estrous cycle increases from a low when a mature corpus luteum is present to a high when follicles are fully grown at estrus, and ovarian weight will then decrease following ovulation (Wesson and Ginther, 1981).

The ovary has a gametogenic and an endocrine role. There are two principal ovarian components, the follicles and corpus luteum. The follicles are important for the production of the ova and synthesis of estrogens, while the corpus luteum is responsible for the production of progestins. Follicles grow and secrete estrogens about every 10

days in mares with cyclic ovarian activity (Van Rensburg and van Niekerk, 1968; Belin et al., 2000).

Follicular Growth

At any one given time in the mares' ovary, there are several follicles of varying size and stages of growth. Folliculogenesis is the process of ovarian follicles developing from primordial to primary, to secondary and then finally antral follicles (Senger, 1997). Primordial follicles are the smallest and most immature structures in the ovay. The primordial follicle develops into the primary follicle. Primary follicles have a single layer of cuboidal follicular cells surrounding the primary oocyte which will start to increase in diameter as follicular growth occurs. This primary follicle either degenerates or continues its growth into a secondary follicle. Secondary follicles have two or more layers of follicular cells around the oocyte. An antral follicle, also known as a tertiary follicle, has an antrum filled with follicular fluid. The enlarged oocyte, which is now enclosed in the acellular zona pellucida, is attached to the inner lining, the membrana granulosa, of the follicle.

Tertiary follicles have three cell layers: the theca externa, theca interna, and granulosa cell layer. The theca externa consists of loose connective tissue that supports the follicle. The theca interna is below the theca externa, these cells synthesize androgens under the influence of LH (Senger, 1997). Granulosa cells possess receptors to FSH and LH (Fay and Douglas, 1987) and synthesize estrogen, inhibin and are bathed in follicular fluid (Senger, 1997). In the mare, antral follicles are usually grouped by size. Small, medium and large classifications consist of follicles measuring less than 10

mm, 10 to 20 mm and greater than 20 mm in diameter, respectively (Senger, 1997). In the mare, these preovulatory graafian follicles range in size from 35 to 55 mm.

The dominant follicle has been detected at emergence (6 mm) close to 1 day earlier than the subordinate follicle. Also, on the day of its emergence, the future dominant follicle was larger than the future subordinate follicle (Gastal et al., 1997). This future dominant follicle will continue to grow to become the ovulatory follicle while the subordinate follicle will regress and undergo atresia. It has been observed that ovulation occurs about 7 days after the initiation of corpus luteum regression, while in some instances mares ovulated during the luteal phase of the cycle or while the corpus luteum was still regressing (Stabenfeldt et al., 1972). Several studies have indicated a greater frequency of ovulation from the left ovary of maiden mares, but after their first pregnancy, the asymmetry is lost (Ginther, 1979). Throughout the ovulatory cycle, significant changes in diameter of largest follicle and second largest subordinate follicle and numbers of follicles 2 to 5 mm, 16 to 20 mm and > 20 mm occur (Ginther and Pierson, 1989). The number of large follicles increases from January to a peak in April and then decreases from May through July, after this there is another peak in August, and then another decline (Wesson and Ginther, 1981).

Once the dominant follicle ovulates, a corpus hemorrhagicum fills the follicular cavity until a corpus luteum forms and becomes functional, which occurs within 5 days of ovulation. The corpus luteum is a structure that, unlike most species, cannot be felt via rectal palpation because of the layer inversion of the mares' ovary. The corpus luteum produces progesterone and aids in the maintenance of pregnancy until the placenta takes over. The average life span of the corpus luteum during the estrous cycle is 12 days (Van

Rensburg and van Niekerk, 1968). The following section will discuss the selection process that follicles undergo to become a dominant follicle.

Follicle Selection

Gonadotropins (LH and FSH) and growth factors regulate selection and maturation of dominant ovarian follicles (Hammond et al., 1991; Adashi 1994; Monniaux et al., 1997). In 1975, it was suggested that the mare expresses two waves of follicular activity during the estrous cycle (Evans and Irvine, 1975). Others proposed that only a single major follicular wave during the equine estrous cycle existed based on transrectal palpation (Ginther, 1979). With the advent of ultrasonography, Ginther (1990a) found that follicular waves (anovulatory and ovulatory) seemed similar to follicular waves in cattle except that follicles in the mare were much larger. Ultrasonic monitoring of individual ovarian follicles in heifers has indicated that there are 2 (Ginther and Pierson, 1988; Ginther et al., 1989) or 3 (Savio et al., 1988; Sirois and Fortune, 1988) waves of follicular activity during most estrous cycles. Breed differences in follicular wave patterns during the estrous cycle have been found in mares (for review see Ginther, 1992) and in cattle (Alvarez et al., 2000). Usually one major wave develops in late diestrus and culminates in the ovulation during estrus in breeds such as quarter horses and ponies. In other breeds such as thoroughbreds, there is a secondary wave, which develops in early diestrus, and like in cattle, may be anovulatory or ovulatory. Recruitment, selection, dominance and atresia are all segments of the dynamics of antral follicles. Recruitment is the stage of antral follicle development in which a group of small follicles begin to develop and produce estradiol. Some of these recruited follicles will undergo atresia.

The follicles that do not undergo atresia have been "selected". In cattle and humans, these follicles will continue their growth and become follicles that produce increasing amounts of estrogen and inhibin. One of these follicles will become the "dominant" follicle that inhibits growth of the other antral follicles and causes them to undergo atresia. In most domestic large animals, recruited follicles will have high follicular fluid content of FSH, low LH and no inhibin (Senger, 1997). Selected follicles exhibit moderate levels of FSH, moderate LH and low inhibin (Senger, 1997). The dominant follicle has low FSH, high LH and high inhibin (Senger, 1997). Goudet et al., (1999) found in the mare that the amount of α-inhibin was lower in 5 to 9 mm follicles than in larger ones. Bergfelt et al., (1991) and Roser et al., (1994) reported a reciprocal relationship between circulating concentrations of inhibin and FSH during the estrous cycle of the mare.

The mechanism involved in the selection of the dominant follicle in monovular species is under intense investigation and is not clearly understood, although estradiol has been suggested as a facilitator of deviation in cattle (Ginther et al., 1996). In the mare, the follicular wave begins with 6 mm follicles during mid-diestrus and these follicles continue to grow similarly until the largest follicle reaches about 23 mm (Gastal et al., 1997). This follicle has been selected to deviate; selection is not complete until deviation has occurred. Deviation is where the growth rates between one follicle and the others differ (Gastal et al., 1999b). Gastal et al., (1997) found that the future dominant follicle emerged one day earlier than did the future subordinate follicle, growth rates did not differ, and that at the beginning of deviation the future dominant follicle was 3 mm larger than the future subordinate follicle. Gastal et al., (1999b) also found that there was a

difference in intrafollicular estradiol concentrations between the two follicles before the difference in diameter and that there is an increase in systemic estradiol the day before the detection of follicle deviation. An increase in the number of LH receptors in granulosa and thecal cells in the dominant follicle has been proposed as a major feature in the process of follicle selection and dominance at least in cattle (Xu Z et al., 1995; Bodensteiner et al., 1996; Stewart et al., 1996; Evans and Fortune, 1997;). Also in cattle, estradiol may function as the mechanism of deviation by inhibiting systemic FSH concentrations and by intrafollicular facilitation of LH responsiveness of the larger follicle (Ginther et al., 1996). Ginther et al., (1999) found that systemic FSH concentrations were decreased for 10 h after the time of deviation in cattle. The continued growth of the dominant follicle appears to be dependent on LH at least in cattle (Ginther et al., 1996; Gong et al., 1996). Gastal et al., (2000) found in pony mares that systemic LH concentrations increased after Day 14 which was similar to reports in mature mares (Gastal et al., 1997; Gastal et al., 1999a) and in cattle (Ginther et al., 1998), and that this increase in LH concentrations preceded deviation. It has been suggested that LH does not play a role in the initiation of deviation, but is involved in subsequent growth of the dominant follicle in the mare (Gastal et al., 2000) and cow (Ginther et al., 1998). Follicular dominance will be reviewed in the following paragraphs.

Follicle Dominance

The dominant follicle of each wave causes atresia of the subordinate follicles and blocks the emergence of the second wave until the dominant follicle approaches ovulation. The next wave can contribute to the regression of the dominant follicle of the

previous wave. Inhibin and estrogen may be the suppressing factors (Ginther et al., 1996). In the mare, Gastal et al., (1999a) found that if the larger follicle is removed at the time of deviation, the smaller follicle is capable of becoming the dominant follicle. Large equine follicles, > 20 mm, can cause a decrease in FSH concentration (Gastal et al., 1999a) and as mentioned have been postulated to play a role in follicle deviation in the cattle (Ginther et al., 1996). Because ablation of the larger follicle increased mean LH concentrations, large follicles of the mare may produce a substance that depresses LH concentrations (Gastal et al., 1999a). Ovarian hormone secretion and follicle development, without alteration of peripheral concentrations of FSH, are inhibited when sheep (Campbell et al., 1991) and cattle (Law et al., 1992) are treated with inhibin-free ovine or bovine follicular fluid, suggesting that factors other than FSH are involved in follicular development.

The levels of IGF-I and IGFBP-3 found in follicular fluid are similar in dominant and subordinate follicles (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996). However, follicular fluid IGFBP-2, -4, and -5 levels are decreased in dominant follicles of cattle (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996). By regulating the bioavailability of IGF-I in the follicle, IGFBPs may play a role in follicular dominance (Stewart et al., 1996). An increase in the number of thecal LH/hCG binding sites along with a decreased amount of IGFBP activity in early dominant follicles was observed in cycling dairy cows; IGF-I may mediate the increase in thecal LH receptors (Stewart et al., 1995; Stewart et al., 1996). The stimulatory actions of IGF-I may be blocked by an increase in IGFBPs in the late dominant follicle and may lead to atresia (Stewart et al., 1996; Spicer et al., 1997). Because a decrease in IGFBP-2 mRNA occurs

during growth of large antral follicles (Howard and Ford, 1992; Samaras et al., 1993;Yuan et al., 1996), changes in local production of IGFBPs likely account for changes in IGFBP levels in follicular fluid. However, IGFBP proteases may also be involved. These topics will be addressed in more detail in a later section.

Growth and Development of the CL

A corpus luteum (CL) is formed from the remaining cells of the ovulated follicle. During the estrous cycle of the mare, the CL occasionally will have prolonged maintenance (Ginther, 1990b), occurring more often at the end of the ovulatory season (Weedman et al., 1993). As mentioned earlier, the average CL lifespan is about 12 days (Stabenfeldt et al., 1972). The primary function of the CL is the synthesis and secretion of progesterone. Short (1962) found that large amounts of progesterone, 20α-hydroxypregn-4-en-3-one, a small quantity of 17α-hydroxyprogesterone, and no C18 or C19 steroids were contained in luteal tissue of the mare. There appears to be a direct and indirect regulation of progesterone secretion by gonadotropins (Kelley et al., 1988). Peripheral plasma levels of progestins are low during the follicular phase, increase within 24 h of ovulation, are increased during the luteal phase, and decline with CL regression (Stabenfeldt et al., 1972). The cyclic progestin levels in the mare (Stabenfeldt et al., 1972) closely resemble those of the cow (Stabenfeldt et al., 1969b) and sow (Stabenfeldt et al., 1969a).

There are two distinct steroidogenic cell types known as the small and large luteal cells which are found in the CL of several species (Ursely and Leymari, 1979; Fitz et al., 1982; Hoyer et al., 1984,1986) including the mare (Kelley et al., 1988; Watson and

Sertich, 1990). The CL is composed of large luteal cells, small luteal cells, a few spindle-shaped endothelial cells, red blood cells, and a small number of leucocytes (Watson and Sterich, 1990). Harrison (1946) thought that the CL in the mare was derived from granulosa cells. Data indicate that large luteal cells of the early CL in cattle, originate from granulosa cells while small luteal cells originate from theca cells (Alila and Hansel, 1984). There are very few large cells present early in the estrous cycle, but the number increases to a peak on Day 12 when they comprise 45% of the steroidogenic cells in ewes (Schwall et al., 1986). Granulosa derived large luteal cells disappear during early pregnancy while cells of thecal origin persist throughout pregnancy in cattle (Alila and Hansel, 1984). In early, mid- and late diestrus, luteal cells from the CL release prostaglandins; these concentrations decrease as the age of the CL increases (Watson and Sterich, 1990). Large luteal cells are thought to secrete high basal amounts of progesterone, are unresponsive to LH and derived from granulosa cells, while small luteal cells have low basal secretion of progesterone, but respond to LH with a dramatic increase in steroid secretion, and are thought to be derived from thecal cells (Koos and Hansel, 1981; Fitz et al., 1982). Large and small cells are interspersed in ruminants, while in the mare the small cells lie between the large cells (Broadley et al., 1994). It was suggested that in the mare CL, large cells bind LH (Broadley et al., 1994).

The CL of the mare produces estrogen if there is an adequate amount of androgen substrate available (Watson and Thomson, 1996). Previously it was thought that the CL of the mare, like that of the ewe and cow, does not produce estrogen (Short, 1964; Mahajan and Samuels, 1974). However, estradiol was detected in the mid-luteal CL of the mare (YoungLai, 1971) thus, being similar to primates (Stouffer et al., 1980). The

equine CL may be involved in estrogen secretion as observed from the CL of non-pregnant and pregnant mares (Montavon et al., 1990; Stabenfeldt et al., 1990). Recently, Rodger et al., (1998) reported that P450C17, the enzyme needed for synthesis of estradiol-17β, is not found in the CL of the non-pregnant mare. Further studies are needed to clarify the role of the CL in estradiol secretion in the mare.

$PGF_{2\alpha}$ is known to play a part in luteolysis at the end of diestrus in mares and other domestic species. In most species PGF1 is the predominant prostaglandin released by the CL, however in the mare, PGE2 has been found to be the predominant prostaglandin (Watson and Sertich, 1990). The mare is more sensitive to prostaglandins than the cow and ewe because the lungs of the mare can only metabolize 30% of the $PGF_{2\alpha}$ in a single passage. Watson and Sertich (1990) found that luteal cells from the CL of the mare released $PGF_{2\alpha}$ during early, mid- and late diestrus, and as CL age increased, $PGF_{2\alpha}$ concentrations decreased. Transportation of the luteolytic uterine $PGF_{2\alpha}$ in the mare is not like that of other species; the $PGF_{2\alpha}$ is not exerted through the local vascular pathway from the uterus but through systemic circulation (Ginther, 1974). Cultured cells from late CL of mares had a decreased synthesis of $PGF_{2\alpha}$, which suggests locally produced $PGF_{2\alpha}$ is not the primary cause of luteolysis in this species (Watson and Sertich, 1990). In the mare, $PGF_{2\alpha}$ concentrations increase in uterine venous blood at the time of luteal regression well before the decline in serum progesterone values (Douglas and Ginther, 1976). There is suggestion that estradiol stimulates endometrial $PGF_{2\alpha}$ production linking it to the luteolytic process in mares (King and Evans, 1988). Hormonal changes in the mares' estrous cycle will be reviewed in this next section.

Endocrine Changes during the Estrous Cycle

Estradiol and progesterone are reciprocally related, estradiol is active during estrus, the follicular phase and progesterone is dominant during diestrus, the luteal phase. LH and FSH are also reciprocally related, however during the few days encompassing ovulation, both levels are significantly elevated (Miller et al., 1980). Estradiol and LH have a similar increasing and decreasing pattern of secretion, although, estradiol peaks a few days before LH. Progesterone increases significantly one day prior to a decrease in LH at the beginning of diestrus and at the end of diestrus; progesterone decreases 2 days before an increase in LH (Nett et al., 1976). The decline in FSH prior to ovulation corresponds to the rise in estradiol levels. FSH increases at the same time estradiol decreases before progesterone increases at the end of estrus. At the end of diestrus, progesterone decreases more rapidly than FSH. Inhibin is thought to reduce FSH secretion. A significant decrease in inhibin concentrations is associated with an increase in FSH and vice versa (Bergfelt et al., 1991). A rise in LH, with a decline in FSH, is associated with the final growth and maturation of the dominant ovulatory follicle (Freedman et al., 1979). The subsequent section will review the changes in gonadotropins during the estrous cycle of the mare.

Gonadotropins

FSH and LH are gonadotropins secreted by the hypophysis that play a major role in follicular growth and development. Ovarian steroidogenesis in granulosa and theca cells is stimulated by gonadotropins through the activation of cAMP. As a result, there is an increase of steroids, especially estradiol-17β, in plasma and follicular fluid of antral follicles. Estradiol secretion from follicles is dependent upon FSH and LH action on

granulosa and thecal cells, respectively. There have been extensive reviews on the actions of gonadotropins in granulosa and thecal cells (Hsueh et al., 1984; Hillier et al., 1995).

In mares, the concentration of LH is minimal during the anovulatory season (Garcia and Ginther, 1976); however, during the ovulatory season the concentrations are high only during estrus and are low during diestrus (Whitmore et al., 1973). The mare exhibits a longer LH surge, longer period of follicular growth and a longer estrus than most other species (Evans and Irvine, 1975). LH may have a luteotrophic role as indicated by the peak serum levels of LH occurring 1 to 2 days after ovulation (Evans and Irvine, 1975). By the onset of estrus LH levels are increasing, reaching their maximum 1 to 2 days after ovulation (Evans and Irvine, 1975) and then decreases over the next 4 to 6 days to the low diestrus values. Fay and Douglas (1987) found that plasma LH concentrations were elevated when progesterone concentrations dropped below 1 ng/ml, and that LH concentrations continued to increase until the day of or the day after ovulation. The follicular fluid concentrations of LH are found to be higher in presumptive ovulatory follicles on the 4th day of estrus (Fay and Douglas, 1987). An increase in thecal LH receptors in presumptive ovulatory follicles is thought to be important in the development of an ovulatory follicle since the increase in LH receptor in the theca allows for increased responsiveness to basal LH concentrations permitting an increase in androgen biosynthesis that could then be aromatized into estrogen (Fay and Douglas, 1987). LH receptor mRNA levels also increase in granulosa cells as the largest follicle reaches the expected time of deviation in cattle (Bodensteiner et al., 1996, Evans and Fortune, 1997) and the mare (Goudet et al., 1999). As the dominant follicle grows,

numbers of LH receptors in granulose cells increase (Stewart et al., 1996). The increase in LH receptors in granulosa cells may account for an enhanced oocyte competence and responsiveness to the impending LH surge.

Evans and Irvine (1975) found that FSH concentrations were raised five-fold by surges occurring at 10 to 11 day intervals during the estrous cycle of the mare. A late estrus surge of FSH initiated the development of up to 20 follicles, while the mid-diestrus FSH surge may be needed for the development of follicles destined to ovulate 10-13 days later (Evans and Irvine, 1975). In agreement with this observation, concentrations of FSH increase after the future dominant follicle reaches 6 mm, peak 3 days later, and then decline (Gastal et al., 1997). Plasma FSH levels were elevated in mares between late estrus and early diestrus, reaching a maximum of 10 ng/ml between Days 5 and 10 after ovulation (Fay and Douglas, 1987). Before the day of the FSH peak, FSH and LH are closely associated, but once the peak occurs, become disassociated until ovulation (Gastal et al., 1997). In cattle, before ovulation there is a concomitant FSH and LH surge (Hansel and Convey, 1983), however, this is not found to occur in mares (Ginther, 1992). Gastal et al. (1997) found that follicular deviation in the mare occurred during high LH and low FSH concentrations. LH is not involved in the initiation of deviation, but may play a role in the subsequent growth (Gastal et al., 2000) and ovulation (Ginther, 1990a) of the dominant follicle. Steroids involved in the estrous cycle of the mare will be discussed in the subsequent section.

Steroids

In the mare the steroidogenic role of the ovaries change drastically during different stages of the estrous cycle. In general, the follicles are associated with the production of estrogens, while the CL is associated with production of progestins.

As mentioned earlier, Short (1962), found that progesterone was the major steroid in the CL of the mare. Progesterone from the CL is responsible for maintenance of early pregnancy up to around 150 days in mares. In 1957, Short was the first to report progesterone production from the equine placenta. From 150 days until term the main source of progesterone (progesterone metabolites: 5α-pregnane metabolite, 20α-dihydroprogesterone, 17α-hydroxyprogesterone) is the placenta (Holtan et al., 1975; Barnes et al., 1975). In cattle and sheep (Donaldson et al., 1970; Katongole et al., 1973; McNatty et al., 1973), systemic progesterone concentrations do not increase until day 3 after ovulation, but begin to increase immediately after ovulation in the mare (Stabenfeldt et al., 1972; Plotka et al., 1975) and swine (Hansel et al., 1973). Progesterone concentrations of mare serum during estrus has been well studied (Hughes et al., 1972; Sharp and Black, 1973; Holtan et al., 1973). Twenty- four hours before ovulation through 8 h after ovulation progesterone concentrations ranged between 60 and 100 pg/ml (Plotka et al., 1975). The concentrations increased 10 h after ovulation to 140 pg/ml and reached 346 pg/ml by 26 h after ovulation (Plotka et al., 1975). Evans and Irvine (1975) found that progesterone levels during estrus were usually less than 0.5 ng/ml and increased after the end of estrus reaching a peak level between 5 and 14 ng/ml 7 to 10 days later. Between day 15 and 16 of the estrous cycle, period when the CL regresses, progesterone concentrations in plasma decrease to below 1 ng/ml (Fay and

Douglas, 1987). Follicular fluid progesterone concentrations did not differ among mares in the late luteal phase, in early estrus, or in late estrus (Short, 1961; Fay and Douglas, 1987; Sirois et al., 1990; Spicer et al., 1991). In atretic follicles, the level of progesterone is lower than those in viable follicles (Kenney et al., 1979). Watson and Hinrich (1988) found that FF progesterone levels were significantly higher in mares with a 35 mm follicle given hCG than mares not given hCG.

Estrogen causes the mare to show behavioral signs of receptivity to a stallion. In FF of the mare, estradiol-17β is the major estrogen found (Short, 1962). Close to the beginning of estrus, circulating concentrations of estrone and estradiol-17β increase, reaching a peak approximately 1 day before ovulation (Meinecke et al., 1987). Estradiol levels then begin to decrease and reach a basal diestrus level near the end of estrus (Fay and Douglas, 1987). Maximal concentrations of plasma estradiol-17β range from 11.5 to 141 pg/ml during estrus (Ginther, 1979; Nelson et al., 1985). The high levels of estrogen will cause a mare's uterus to become flaccid while in heat, which is different from other farm species. Short (1962) suggested that the theca interna cells that line the graafian follicle of the mare convert progesterone to estradiol-17β and was later confirmed by Hay et al., (1975). Estradiol concentrations in FF increase from early to late estrus (Spicer et al., 1991), and content of total estrogen is lower in atretic follicles than in viable follicles (Kenney et al., 1979). There is a 3-fold increase in estrogen and androgen in follicles with a 3 cm diameter; this appears to be the turning point in the maturation of the equine follicle (Kenney et al., 1979). Fay and Douglas (1987) showed that the presumptive ovulatory follicle has estradiol concentrations between 30- and 50- fold higher than that in non-ovulatory follicles. More recent studies show that systemic estradiol levels

increased the day before the detection of follicle deviation (Gastal et al., 1999b). Gastal et al., (1999a) found that as the larger follicle reached > 20 mm, it was responsible for the increase in systemic estradiol concentrations. King and Evans (1988) suggested that estradiol stimulation of endometrial $PGF_{2\alpha}$ production may be the link to the luteolytic process in the mare and that estradiol production is the result of luteal phase follicular growth.

As ovulation approaches, in addition to increases in estradiol and progesterone in FF, the follicular content of androstenedione (Meinecke et al., 1987; Fay and Douglas, 1987) and testosterone (Watson and Hinrich, 1988; Spicer et al., 1991) increases. Follicular fluid of the mare contains mainly androstenedione with lesser amounts of testosterone, epitestosterone, and 19-norandrostenedione (Short, 1961; Spicer et al., 1991). Regardless of follicle size or status there is a relationship between LH stimulation of androgen biosynthesis *in vivo* and *in vitro* (Fortune and Armstrong, 1977; Fay and Douglas, 1987). There is evidence in the mare and in other species, that androgens are produced by the theca interna cells under the control of LH (Ginther, 1979; McNatty et al., 1984; Tsang et al., 1985; Stewart et al., 1995). Plasma androstenedione concentrations remain constant during late diestrus and early estrus then increase significantly by the 4th day of estrus (Fay and Douglas, 1987). Levels of testosterone in plasma are elevated during mid-late diestrus, fall to basal concentrations at the beginning of estrus, and rise again during late diestrus (Fay and Douglas, 1987). From these findings, one could speculate that androstenedione plays an important regulatory role in the growth and development of large, ovulatory follicles, since androgens cause an increase in FSH production (Thompson et al., 1991). The levels of 17β-

hydroxyandrogens (testosterone, dihydrotestosterone, 3α- and 3β-androstandiols and androstenediols) are lower in atretic than viable follicles (Kenney et al., 1979).

ROLE OF IGF AND IGFBP IN FOLLICULAR DEVELOPMENT

IGFs in Follicular Steroidogenesis and mitogenesis

Insulin-like growth factors (IGF-I and -II) are single polypeptides that consist of an intact C-peptide and three intramolecular disulfide bonds that are structurally similar to proinsulin. Amino acid sequences of IGF-I and -II have high homology across species; bovine IGF-I is 99%, 100%, 100% and 96% homologous to ovine, porcine, human and rat, respectively (for review see, Spicer and Echternkamp, 1995). Also, bovine IGF-II is 99%, 97%, 96% and 96 % homologous to the ovine, porcine, human and rat, respectively (for review see, Spicer and Echternkamp, 1995).

The presence of IGFs in the ovary was first reported by Hammond et al., (1982; 1985). Subsequently, IGF-I and/or -II have been found in the follicular fluid of cattle (Spicer et al., 1988), sheep (Spicer et al., 1995), horses (Spicer et al., 1991) and humans (Jesionowska et al., 1990). IGF-I and IGF-II mRNA has been found in ovarian tissue of rats, women, cattle, pigs, and sheep including granulosa, thecal, stromal, and luteal cells (Murphy et al., 1987; Einspanier et al., 1990; Barecca et al., 1993; Samaras et al., 1994; Spicer et al., 1995). These latter observations suggest that IGF-I and -II are locally produced in the ovary. In follicular fluid of pigs (Hammond et al., 1988a) and cattle (Spicer et al., 1988), concentrations of IGF-I increase as follicle size increases. In mares,

IGF-I concentrations in follicular fluid or serum did not change from early to late estrus (Spicer et al., 1991). Follicle diameter was significantly positively correlated ($r = 0.2$ to 0.7) with FF IGF-I concentrations in mares (Spicer et al., 1991), beef cows (Spicer et al., 1988), and prepubertal gilts (Spicer et al., 1992; Echternkamp et al., 1994b; Hammond et al., 1988a). In comparison, FF IGF-II concentrations were approximately 1.5-fold higher in small (atretic) than in large normal or atretic follicles of ewes (Monget et al., 1993) and in cattle (Spicer and Echternkamp, 1995; Stewart et al., 1996). However, IGF-II levels in FF did not differ between small and large healthy follicles of ewes (Spicer et al., 1995) or gilts (Spicer et al., 1992). Collectively, these results indicate that intraovarian production of IGF-I and IGF-II are controlled differently and may account for their changes in FF.

Receptors for IGF and its mRNA have been found in many ovarian cells including granulosa cells (Baranao and Hammond, 1984; Spicer et al., 1994a), thecal cells (Hernandez et al., 1988a; El-Roeiy et al., 1993; Stewart et al., 1995), luteal cells (Ladenheim et al., 1984; Samoto et al., 1993), and ovarian stromal cells (Jarrett et al., 1985; Hernandez et al., 1992). In cultured rat granulosa cells, addition of FSH and LH increase numbers of IGF-I receptors nearly three-fold (Adashi et al., 1986, 1988), and supplementation of IGF-I increases LH receptor mRNA levels in granulosa and theca cells (Adashi et al., 1985; Cara et al., 1990; Magoffin and Weitsman, 1994; Hirakawa et al., 1999) and FSH receptor mRNA levels in granulosa cells (Zhou et al., 1997; Minegishi et al., 2000). Spicer et al., (1994b) found that FSH increased numbers of IGF-I receptors in bovine granulosa cells of small follicles (but not large) and that granulosa cells from large follicles have greater numbers of IGF-I receptors than small follicles. In cultured bovine thecal cells, LH had no effect on the number of IGF-I receptors whereas

IGF-I increased the number of LH receptors. This heterologous upregulation between IGF-I and gonadotropins may explain their synergistic effects on steroidogenesis (Stewart et al., 1995). IGF-I has been shown to have stimulatory effects on steroidogenesis and mitogenesis in cultured granulosa and thecal cells from several species including rats (Adashi et al., 1985; Hernandez et al., 1988b; Cara and Rosenfield, 1988), pigs (Hammond et al., 1988a) and cattle (Spicer et al., 1993; Stewart et al., 1995). Granulosa and theca cell proliferation and differentiation are stimulated by IGF-I and IGF-II (Hammond et al., 1991; Adashi, 1994; Monniaux et al., 1997). In the absence of gonadotropins, IGF-I stimulates mitogenesis of granulosa and thecal cells, while with gonadotropins, IGF-I synergistically stimulates steroidogenesis of granulosa and thecal cells (Giudice, 1992; Spicer and Echternkamp, 1995).

IGF-I and IGF-II are bound to high affinity binding proteins (IGFBPs) in biological fluids; these binding proteins are regulators of IGF bioavailability for the target cells (Zapf, 1995; Jones and Clemmons, 1995). Currently there are at least six IGFBPs that have been cloned and sequenced: IGFBP-1, -2, -3, -4, -5 and -6 (for review see, Rechler, 1993; Jones and Clemmons, 1995; Spicer and Echternkamp, 1995). The following sections will first describe how these IGFBPs change during ovarian follicular development and then describe the mechanisms by which these changes in IGFBP may occur.

Changes in Follicular Fluid IGFBPs during Follicular Development

IGFBP-1 was first detected in human FF in 1984 (Seppala et al., 1984). In humans, IGFBP-1 is produced by theca and stromal explants cultures, as well as

granulosa cell cultures under basal conditions (Mason et al., 1993). IGFBP-1 mRNA was found in granulosa cells of dominant follicles of women (El-Roeiy et al., 1994). Whereas it is not detectable in the rat (Nakatani et al., 1991) or porcine (Hammond et al., 1991) ovary. Purified human IGFBP-1 inhibits FSH-induced estradiol and progesterone production and IGF-I induced progesterone production in human granulosa cells (Mason et al., 1992; Hillensjo et al., 1992; Iwashita et al., 1994). Schams et al., (1999) has found evidence for the local expression of IGFBP-1 in the theca interna cell and granulosa cell compartment during the final growth of bovine follicles. In the mare, IGFBP-1 is thought to be found at the 28-32 kDa band region in FF (Gerard and Monget, 1998), but the presence of IGFBP-1 in FF of the mare and cow awaits verification.

The presence of IGFBP-2 in FF was first reported in pigs in 1991 (Mondschein et al., 1991), humans in 1992 (Cataldo and Giudice, 1992), and in cattle in 1994 (Echternkamp et al., 1994a; Stanko et al., 1994). In ovarian follicular fluid of pigs, cattle, horses, and sheep (Spicer and Echternkamp, 1995; Echternkamp et al., 1994ab; Stewart et al., 1996; Spicer et al., 1995), IGFBP-2 is the second most prevalent IGFBP. Atretic follicles contain more IGFBP-2 than do healthy follicles in sheep (Monget et al., 1993), cattle (Echternkamp et al., 1994a), and women (Cataldo and Giudice, 1992; San Roman and Magoffin, 1993). IGFBP-2 levels are greater in small than in medium or large follicles in sheep (Spicer et al., 1995), pigs (Mondschein et al., 1991; Echternkamp et al., 1994b) and cattle (Echternkamp et al., 1994a). In ovine preovulatory follicles, in situ hybridization experiments demonstrated that IGFBP-2 is expressed in granulosa cells (Besnard et al., 1996a). Dominant follicles of women express IGFBP-2 mRNA (El-Roeiy et al., 1994) and there are high amounts of IGFBP-2 mRNA found in the theca

cells of dominant follicles in rats (Nakatani et al, 1991). In follicular fluid of dominant follicles of cattle, the amount of IGFBP-2 decreases compared with subordinate follicles (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996). In the mare, the binding activity of intrafollicular IGFBP-2 decreases during growth of the dominant follicle and is elevated in subordinate follicles (Gerard and Monget, 1998). These changes may be due to changes in local synthesis since ovine follicles have decreased IGFBP-2 mRNA in large healthy versus atretic follicles (Besnard et al., 1996a) and porcine dominant follicles contain IGFBP-2 mRNA (Yuan et al., 1996). In cattle, IGFBP-2 mRNA from granulosa cells was nearly undetectable in dominant follicles, whereas in subordinate follicles there was an abundance of IGFBP-2 mRNA (Yuan et al., 1998). Follicular fluid levels of IGFBP-2 are negatively correlated to follicular fluid levels of estradiol and androstenedione in cattle (Echternkamp et al., 1994a; Stewart et al., 1996) and sheep (Spicer et al., 1995), and suggest IGFBP-2 may inhibit steroidogenesis. Indeed, Spicer et al., (1997) found that IGF-I induced androstenedione production was weakly inhibited by IGFBP-2 in bovine thecal cells, however, LH-induced androstenedione production in the absence of IGF-I was not effected by IGFBP-2.

The 42-44-kDa IGFBP, IGFBP-3, is the major IGF-binding protein in FF and its levels do not vary with physiological status of the follicle of mares (Gerard and Monget, 1998), sows (Howard and Ford, 1992), women (Cataldo and Giudice, 1992; San Roman and Magoffin, 1993), and cows (Echternkamp et al., 1994a; Funston et al., 1996). The detection of IGFBP-3 mRNA within porcine (Zhou et al., 1996), ovine (Perks and Wathes, 1996), and bovine (Yuan et al., 1998) follicles using in situ hybridization have

not been successful, although porcine granulosa cells produced IGFBP-3 *in vitro* (Grimes and Hammond, 1994). El-Roeiy et al., (1994) found mRNA for IGFBP-3 to be present only in the theca layer of small, androgenic follicles of women. However, in dominant follicles, mRNA for IGFBP-3 is found in both theca and granulosa cells (El-Roeiy et al., 1994). In the rat ovary, IGFBP-3 mRNA is localized to corpora lutea (Erickson et al., 1992). For cattle it was hypothesized that the source of FF IGFBP-3 (Echternkamp et al., 1994a) is transudation from blood plasma and not follicular IGFBP-3 synthesis, which Yuan et al., (1998) confirmed. Whether this is the case for mares will require further study.

In bovine thecal cells, IGFBP-3 had no effect on LH-induced androstenedione or progesterone production (Spicer et al., 1997). However, the stimulatory effects of IGF-I on progesterone and androstenedione production were reduced by IGFBP-3 (Spicer et al., 1997). Similarly, recombinant human IGFBP-3 inhibited FSH-induced estradiol and progesterone production and IGF-I-induced amino acid uptake but had no effect on FSH-induced progesterone production by human granulosa cells (Mason et al., 1992; Hillensjo et al., 1992; Iwashita et al., 1994) or bovine granulosa cells (Spicer and Chamberlain, 1999). In rodents, IGFBP-3 attenuated the inhibitory effect of hCG and IGF-I on follicular cell apoptosis (Chun et al., 1994) and inhibited FSH from suppressing apoptosis (Flaws et al., 1995). Thus, IGFBP-3 inhibits IGF-I stimulated functions of granulosa and thecal cells of several species.

The smallest (i.e., 24-kDa) and least prevalent IGFBP in FF of all species studied to date, including the mare, is IGFBP-4 (Gerard and Monget, 1998). In follicular fluid of dominant follicles from cattle, the amount of IGFBP-4 decreased (Stewart et al., 1996; de

la Sota et al., 1996; Funston et al., 1996), while IGFBP-4 levels increased with atresia of the dominant follicle and in subordinate follicles compared to E_2 active dominant follicles (de la Sota et al., 1996). Funston et al., (1996) observed small follicles to contain IGFBP-4, as determined by immunoprecipitation. Through in situ hybridization, theca cells of ovine preovulatory follicles have been shown to express IGFBP-4 mRNA (Besnard et al., 1996a). During growth of antral follicles in sheep, there is a decrease in the 24-kDa species (Monget et al., 1993). Cataldo and Giudice (1992) reported an increase in IGFBP-4 in the fluid of atretic follicles of women. A 26-kDa, 24-kDa, and a 16.5-kDa band for IGFBP-4 were seen in human FF following immunoblotting (Cwyfan-Hughes et al., 1997). In FF of atretic human follicles, the 24-kDa band was the predominate form of IGFBP-4, while in dominant follicles most of the IGFBP-4 present was fragmented (Cwyfan-Hughes et al., 1997). IGFBP-4 is produced in porcine (Grimes et al., 1994a) and rat granulosa cells (Erickson et al., 1992), whereas in cattle, the expression of IGFBP-4 mRNA is confined to theca tissue (Armstrong et al., 1998). IGFBP-4 mRNA is localized to atretic granulosa cells in the rat ovary (Erickson et al., 1992) and in both the theca and granulosa cell layers of small, androgenic follicles and large dominant follicles of women (El-Roeiy et al., 1994). Therefore, changes in IGFBP-4 levels in follicular fluid during follicular growth and atresia is likely due to changes in local production of IGFBP-4. Also, there is evidence of proteolysis regulating IGFBP-4, which will be reviewed in detail in a later section.

In bovine follicular fluid, a 29- to 32-kDa IGFBP has been tentatively identified as IGFBP-5 by western immunoblotting (Stanko et al., 1994; Funston et al., 1996. Preovulatory follicles of sheep express IGFBP-5 mRNA in thecal cells (Besnard et al.,

1996a). Similar to IGFBP-2 and -4, the amount of IGFBP-5 decreases in follicular fluid of dominant follicles from cattle (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996), however IGFBP-5 levels increase with atresia of the dominant follicle and in subordinate follicles compared to E_2 active dominant follicles (Echternkamp et al., 1994; de la Sota et al., 1996; Stewart et al., 1996). The greatest levels of IGFBP-5 were found in small follicles as determined by using immunoprecipitation (Funston et al., 1996) and ligand blotting (Stewart et al., 1996). During the first follicular wave of the bovine estrous cycle, IGFBP-5 activity was greater in subordinate small (< 6 mm in diameter) and large (≥ 6 mm in diameter) follicles compared with early dominant follicles (Stewart et al., 1996), which has been confirmed by others (Carolan et al., 1996; de la Sota et al., 1996). In FF of some early dominant follicles of mares, the 28-32-kDa IGFBP bands representing IGFBP-5 were present and then were undetectable in the subsequent maturation stages of healthy follicles (Gerard and Monget, 1998). IGFBP-5 mRNA was expressed in the theca and granulosa cell layers of small, androgenic follicles, as well as in large dominant follicles of humans (El-Roeiy et al., 1994). In the rat ovary, mRNA is expressed in atretic granulosa cells, corpora lutea, and surface epithelium (Erickson et al., 1992). Porcine (Grimes et al., 1994a) and rat (Erickson et al., 1992) granulosa cells produce IGFBP-5. Therefore, as with IGFBP-2 and -4, changes in IGFBP-5 levels in FF during follicular growth and atresia is likely due to changes in local production of IGFBP-5. Proteolysis of IGFBP-5 is also a likely possibility that will be discussed in a later section.

Within the ovary, IGFBP-6 gene has been localized in the rat theca-interstitial compartment (Rohan et al., 1993), it appears to be produced by rat granulosa cells.

Human IGFBP-6 mRNA is found in thecal, stromal, and granulosa cells, using RT-PCR (Voutilainen et al., 1996). IGFBP-6 has been identified in porcine follicular fluid (Shimasaki et al., 1991), but because IGFBP-6 is not found in porcine granulosa cells, IGFBP-6 may be produced by other ovarian cells in the pig (Shimasaki et al., 1991).

REGULATION OF IGFBPs

Regulation of Follicular Cell IGFBP Gene Transcription

Proteolysis, posttranslational modification (phosphorylation), differential localization of the IGFBPs (on the cell surface or in the extracellular matrix) are all physiologic processes that result in a change in the affinity of IGFBPs for their ligands thus altering target cell actions (Clemmons, 1993; Jones and Clemmons, 1995).

Because high levels of FSH are found in dominant follicles (McNatty et al., 1975) and granulosa cell production of IGFBPs are inhibited by FSH (Adashi et al., 1991), it has been postulated that IGFBP gene expression in dominant follicles is inhibited by large quantities of FSH (Erickson et al., 1992). The expression of IGFBP-4 mRNA is very profound in atretic cohort follicles (Erickson et al., 1992), and after the preovulatory surge of LH (Smith et al., 1975). Erickson et al., (1992) theorized that IGFBP-4 gene transcription in granulosa cells of cohort graafian follicles are activated by a hormonal response, likely LH, that result in their destruction. In bovine follicles, FSH inhibits the expression of IGFBP-2 mRNA in granulosa cells, while LH enhanced IGFBP-4 mRNA expression in thecal cells (Armstrong et al., 1998).

In multiple ovulators like the pig, mRNA expression for IGFBP-2 and -3 is limited to certain times and cell types throughout ovarian development and the reproductive cycle (Samaras et al., 1992). In immature and preovulatory follicles there was no difference in IGFBP-3 mRNA levels, however, in luteal ovaries there was a 10-fold increase, with no change in IGFBP-2 mRNA levels (Samaras et al., 1992). Granulosa cells have the greatest whereas thecal and luteal cells contain the lowest amount of IGFBP-2 mRNA, while IGFBP-3 mRNA was not detected in granulosa cells (Samaras et al., 1992). Whether these changes occur in single ovulating species like the mare, is unknown, however in the cow, there is an increase in granulosa cell IGFBP-2 mRNA during atresia (Armstrong et al., 1998). What hormones, if any, regulate IGFBP-2 synthesis in the mare is also unknown.

IGFBP-5 mRNA is found in granulosa cells of atretic preantral and antral follicles in rats (Erickson et al., 1992). Because IGFBP-5 mRNA levels in granulosa cells of rats are inhibited by FSH, FSH may be inhibiting IGFBP-4 and -5 gene transcription or increasing IGFBP-4 and -5 mRNA degradation in the rat (Liu et al., 1993). Theca tissue of healthy bovine follicles was found to express IGFBP-4 mRNA, the expression did not change during growth of the follicle (Armstrong et al., 1998). Whether IGFBP-4 and -5 are produced by ovarian cells of mares is unknown and whether IGFBP-5 is produced in ovarian cells of cows is unknown.

Posttranslational modifications of IGFBPs

Through posttranslational changes (e.g. phosphorylation), the affinity of IGFBPs for IGF-I and -II can be modulated (Monget et al., 1996). Phosphorylation of IGFBP-1, -2, -3, and -5 has been shown (Clemmons, 1993). At the end of pregnancy, in human

amniotic fluid the affinity of IGFBP-1 for IGFs was reported to be increased by phosphorylation (Koistinen et al., 1993). Jones et al., (1993a) found that the serine residues 101, 119, and 169 of IGFBP-1 could be post-translationally phosphorylated. Phosphorylated IGFBP-1 is present in adults and has a higher affinity for IGF-I than does the non-phosphorylated form (Jones et al., 1991; Westwood et al., 1997) and phosphorylated forms migrate on 12% SDS-PAGE much faster than do the nonphosphorylated forms (Jones et al., 1991). The affinity of non-phosphorylated IGFBP-1 for IGF-I is similar to that of IGF-I receptor. Phosphorylated IGFBP-1, however, has a greater affinity to IGF-I than does the type 1 IGF receptor, allowing the IGF-I to bind to the binding protein rather than the receptor, inhibiting IGF activity (Westwood, 1999). Glycosylation of IGFBP-3, -4, -5 and -6 occurs (Jones and Clemmons, 1995), IGFBP-4 has a N-linked glycosylation site while IGFBP-5 and -6 do not, but are O-glycosylated (Clemmons, 1993). It is known that IGFBP-1 and -2 are not glycosylated (Clemmons, 1993). The functional significance of glycosylation is undetermined (Jones and Clemmons, 1995). So far, results indicate neither glycosylation nor phosphorylation appear to have much influence on IGF binding affinities of IGFBP-3, -4, -5, and -6 (Hoeck and Mukku, 1994; Coverley and Baxter, 1995).

When IGFBPs are bound to the extracellular matrix, like IGFBP-5 (Jones et al., 1993b), or are proteolysed (IGFBP-3, IGFBP-4, and IGFBP-5), the affinity of IGFBPs for IGFs are reduced (Monget et al., 1996). There is an eightfold reduction in the affinity of IGFBP-5 for IGF-I and a 60% increase in cell growth stimulated by IGF-I when IGFBP-5 is bound to the extracellular matrix (Clemmons, 1993). When IGFBP-3 is

bound to cell surfaces there is an enhancement of IGF action and a 10- to 12-fold decrease in its affinity for IGF-I and IGF-II (Clemmons, 1993).

Proteolysis of IGFBPs

Modulation of IGFBP levels in various tissues has been linked to proteolytic activity, thus affecting IGF bioavailability. Grimes and Hammond (1994) theorized that by cleaving the intact IGFBPs into fragments, the resulting degradation could release bound IGF from the binding proteins, thus leading to follicular growth and maturation. Most of the IGFBPs proteolytic fragments do not bind IGF-I (Rajaram et al., 1997). Extracellular IGF stabilization and fine-tuning of IGF activity could be a result of a decline in IGFBP degradation (Grimes and Hammond, 1994).

A postulated theory about increasing IGF availability involves the interaction of IGFBP-3 and the low molecular weight binding proteins (for review see, Rajaram et al., 1997). The binding of IGF-I to IGFBP-3 is known to increase the half-life of IGF-I from approximately 20 to 30 min in the free form to 15 to 20 h in the bound form (Zapf et al., 1986). IGFs may either remain free or bind to a lower molecular weight IGFBP and cross the endothelium once they are dissociated from IGFBP-3. A protease may degrade the low molecular weight IGFBP once IGF is transported into the tissue space, thus releasing and enabling IGF-I and -II to bind to their respective receptors. Intact IGFBP-3 has been found in follicular compartments; thus, proteolysis can not fully explain how IGF passes the endothelial barrier. During preovulatory maturation in pigs, intrafollicular IGFBP-3 activity is enhanced due to the increased vascularity and permeability of the follicle during follicular growth, as hypothesized by Grimes et al., (1994b). However,

this does not occur in cattle (Echternkamp et al., 1994a). IGFBP proteolysis was originally described in the circulation of pregnant women (Giudice et al., 1990a; Hossenlopp et al., 1990). Modified or cleaved binding proteins are present in most, if not all extra circulatory fluids, as found by subsequent work (Lalou and Binoux, 1993; Xu S et al., 1995; Matsumoto et al., 1996). The following paragraphs will review published evidence for IGFBP proteolysis in ovarian follicular fluid.

Evidence of IGFBP-2 proteolysis exists for bovine (Stanko et al., 1994), ovine (Besnard et al., 1996b; Monget et al., 1996) and porcine (Besnard et al., 1997) follicular fluid, and human thecal- and stromal-conditioned medium (Mason et al., 1996). In ovine, follicular fluid proteolysis of IGFBP-2 is enhanced during terminal follicular growth of antral follicles (Monget et al., 1996). In bovine follicular fluid of dominant and subordinate follicles, two non-IGF binding fragments of IGFBP-2, approximately 22- and 14-kDa, existed along with the intact IGFBP-2 (34-kDa) as detected by immunoblotting (Stanko et al., 1994). In human thecal- and stromal-conditioned medium, detected by immunoblotting, an intact band of IGFBP-2 was reported at 35-kDa as well as several lower molecular weight bands at 33-, 32-, 25-, 23-, 20.5-, and 16-kDa, the latter of which are thought to be products of proteolysis (Mason et al., 1996). However, immunoblotting may not detect all proteolytic fragments of a particular IGFBP and may detect fragments of other IGFBPs.

Proteolytic degradation of IGFBP-2 by ovine follicular fluid from follicles of different classes was inferred after detecting multiple bands of low molecular weights via immunoblotting (Besnard et al., 1996b). There is a slight increase in IGFBP-2 proteolytic activity during ovine follicular growth, as medium follicles have greater

proteolytic activity than in small follicles (Besnard et al., 1996b). Atretic and healthy ovine medium follicles had similar proteolytic activity (Besnard et al., 1996b).

In porcine growing follicles 2 to 7 mm in diameter, proteolytic activity for IGFBP-2 was found in follicular fluid; in atretic follicles there was a marked decrease in IGFBP-2 proteolytic activity, as detected by immunoblotting (Besnard et al., 1997). A 16-kDa fragment was detected by immunoblotting porcine preovulatory follicular fluid incubated with recombinant human IGFBP-2 (Besnard et al., 1997). EDTA (a calcium chelator) and 1,10 phenanthroline (a chelator of zinc ions) was found to inhibit IGFBP-2 proteolysis of porcine follicular fluid from preovulatory follicles (Besnard et al., 1997). The IGFBP-2 proteolytic activity that was inhibited by EDTA was completely restored by the addition of calcium chloride and zinc chloride (Besnard et al., 1997). The addition of PMSF (a serine protease inhibitor), aprotinin (a serine protease inhibitor), benzamide (a serine protease inhibitor), E64 (a cysteine protease inhibitor), phosphoramidon (an endopeptidase inhibitor), and leupeptin (a trypsin-like serine and cysteine protease inhibitor) in porcine follicular fluid resulted in little or no inhibition of porcine IGFBP-2 proteolysis (Besnard et al., 1997). These studies suggest that the protease(s) involved in the degradation of IGFBP-2 in porcine follicular fluid belong to the metalloprotease family and are calcium- and zinc-dependent (Besnard et al., 1997). Caution should be exerted when interpreting the data because not all IGFBP-2 proteolytic fragments may have been detected by the antisera used during immunoblotting.

Several biological fluids contain IGFBP-3 protease activity, including follicular fluid from women (Giudice et al., 1990b; Hossenlopp et al., 1990; Davies et al., 1991), pigs (Besnard et al., 1997), sheep (Besnard et al., 1996b; Monget et al., 1996), rats

(Davenport et al., 1990; Gargosky et al., 1990), and mouse pregnancy serum (Fielder et al., 1990), and human seminal plasma (Cohen et al., 1992). Proteolytic activity of IGFBP-3 is decreased during terminal follicular growth of antral follicles of sheep (Monget et al., 1996). In small (2-3 mm) ovine follicles, the proteolytic activity of IGFBP-3 increases (Monget et al., 1996). During IGFBP-3 proteolysis, low molecular weight fragments have been detected by Western immunoblots, but as with IGFBP-2, these fragments do not bind IGF-I or -II, and not all fragments may be detected using immunoblotting. Cwyfan-Hughes et al., (1997) detected via immunoblotting, intact IGFBP-3 (41.5- and 44-kDa) as well as a lower molecular weight doublet (30- and 32-kDa) in cultured human thecal explants from dominant follicles. As the size of the dominant follicle increased the proportion of IGFBP-3 appearing as a 30- and 32-kDa doublet increased from 2 to 78% (Cwyfan-Hughes et al., 1997). Also using immunoblotting, ovine follicular fluid from small atretic follicles was found to degrade IGFBP-3, and EDTA and 1,10 phenanthroline blocked the degradation of IGFBP-3 (Besnard et al., 1996b). The addition of calcium chloride partially reversed EDTA's effect and was totally opposed by the addition of both calcium and zinc chloride, and the effects of 1,10 phenanthroline were negated by the addition of zinc chloride (Besnard et al., 1996b). Thus, intrafollicular ovine IGFBP-3 proteolytic activity is similar to that of IGFBP-2 in pigs, in that it involves calcium- and zinc-dependent metalloproteases (Besnard et al., 1996b).

The regulation of IGFBP-3 proteolysis is believed to be hormonally controlled. Diabetes mellitus patients that are non-insulin dependent, were administered insulin and it was found that IGFBP-3 protease activity decreased, suggesting that insulin may have a

role in regulating IGFBP-3 proteolysis (Bang et al., 1994). Conversely, western immunoblots demonstrated that insulin (40% intact IGFBP-3) or EGF (26% intact IGFBP-3) treatments did not have a significant effect on IGFBP-3 proteolysis, when compared to controls (37% intact IGFBP-3) in cultured porcine granulosa cells (Grimes and Hammond, 1994). The addition of IGF-I or -II to porcine granulosa cell cultures diminished the proteolysis of IGFBP-3, however, FSH (29%), estradiol (20%), and GH (23%) did not significantly affect proteolysis (Grimes and Hammond, 1994). Since insulin was ineffective in preventing degradation of IGFBP-3, a non-receptor-mediated action of IGF-I was proposed to regulate IGFBP proteases (Grimes et al., 1994b).

The existence of an ovarian IGFBP-4 protease was first reported in 1994 (Adashi et al., 1994; Erickson et al., 1994). Subsequently, protease activity for IGFBP-4 has been reported in dominant estrogenic follicles of women (Chandrasekher et al., 1995), porcine (Besnard et al., 1997) and ovine follicular fluid (Besnard et al., 1996b; Monget et al., 1994; Mazerbourg et al., 1999), porcine (Cohick et al., 1993) and human (Kamyar et al., 1994) smooth muscle cells, endometrial stromal cells (Irwin et al., 1995) and deciduas (Myers et al., 1993). The IGFBP-4 protease activity in human follicular fluid was pH and temperature dependent (Chandresekher et al., 1995). Protease activity is induced between temperature ranges of 25 to 37°C, with the maximum IGFBP-4 reduction occurring at 37°C (Chandrasekher et al., 1995). A pH range of 7 to 9 produces optimal protease activity (Chandrasekher et al., 1995). Proteolysis of IGFBP-4 is enhanced during terminal follicular growth of antral follicles of sheep (Monget et al., 1996). IGFBP-4 proteolytic activity in large (> 5 mm) follicles decreases during follicular atresia (Monget et al., 1996). The protease degrading IGFBP-4 from porcine follicular fluid

exists in growing, healthy follicles ranging from 2 to 7 mm in diameter, which is similar to porcine IGFBP-2 protease (Besnard et al., 1997). Inhibition of IGFBP-4 degradation in porcine (Besnard et al., 1997) and ovine (Besnard et al., 1996b) follicular fluid samples is possible with the addition of EDTA and 1,10 phenanthroline (Besnard et al., 1997), suggesting that the follicular IGFBP-4 protease is a calcium- and zinc-dependent metalloprotease, which is similar to the follicular fluid protease found to degrade IGFBP-2 and -3 in sheep and pigs. In porcine smooth muscle cells, an intact 24-kDa IGFBP that coincides with nonglycosylated IGFBP-4, is proteolytically cleaved to a 16-kDa fragment which is not capable of binding IGF-I/-II (Cohick et al., 1993). Exposure of these cells to insulin had no effect on IGFBP-4 proteolysis, however exposure to IGF-I will increase the amount of IGFBP-4 fragments (Cohick et al., 1993).

In cultured human granulosa cells, FSH and estradiol have been shown to induce IGFBP-4 proteolysis; IGFBP-4 proteolytic activity in medium was analyzed by an IGFBP-4 protease assay and was assessed by the amount of proteolysis of iodinated IGFBP-4, via SDS-PAGE (Iwashita et al., 1996). It remains unknown if estradiol and FSH directly stimulate the production of a protease by granulosa cells or if it activates proteolytic activity through IGF-mediated mechanisms. Iwashita et al., (1998) found that the addition of IGF-I, but not FSH, to granulosa cell cultures activated the proteolysis of ^{125}I-IGFBP-4, indicating that the binding of IGFs to IGFBP-4 induces the susceptibility of IGFBP-4 to proteolysis, exactly opposite to what was reported for the IGFBP-3 protease produced by porcine granulosa cells (Grimes et al., 1994). IGFBP-4 inhibits IGF-I stimulated estradiol release by rat (Liu et al., 1993) and human (Iwashita et al., 1996) granulosa cells *in vitro*. Also, the addition of IGFBP-4 to rat granulosa cells

cultured in the presence of either 100 or 30 ng/ml of FSH inhibited estradiol production (Ui et al., 1989; Liu et al., 1993). A decrease in intrafollicular IGFBP-4 levels during follicular growth is not associated with a decrease in expression in the corresponding mRNA, and thus the absence of IGFBP-4 protein in follicular fluid from the preovulatory follicles may be due to proteolytic degradation (Fowlkes et al., 1995). IGFBPs in human follicular fluid also influence IGFBP-4 degradation. Cwyfan-Hughes et al., (1997) found that IGFBP-4 protease activity is significantly reduced by adding unsaturated IGFBP-1, -2, -3, or -4, as shown by Western immunoblots. Thus, a reduction in IGF-I levels, whether due to a decrease in its local production or due to an increase in other IGFBPs, may increase IGFBP-4 proteolysis.

Evidence for proteolysis of IGFBP-5 has been found in bovine (Stanko et al., 1994), ovine (Besnard et al., 1996b; Monget et al., 1996) and porcine (Besnard et al., 1997) follicular fluid and serum of neonatal fasted pigs (McCusker et al., 1991), as well as in cultured rat granulosa cells (Fielder et al., 1993), human fibroblasts (Camancho-Hubner et al., 1992), and porcine smooth muscle cells (Cohick et al., 1993). Western immunoblotting detected a 21-kDa fragment in bovine follicular fluid that reacts to the IGFBP-5 antiserum, along with an intact IGFBP-5 doublet (31- and 32-kDa) (Stanko et al., 1994). These fragments coincide with IGFBP-5 fragments found in serum of neonatal fasted pigs (McCusker et al., 1991), and in media conditioned by porcine smooth muscle cells (Cohick et al., 1993) and human fibroblasts (Camancho-Hubner et al., 1992). In bone cells, calcium-dependent serine proteases and matrix metalloproteases degrade IGFBP-5 (Gabbitas et al., 1996).

Using Western immunoblotting techniques, an intact 29-kDa (nonreduced) and 34-kDa (reduced) intact bands for IGFBP-5 are lost and a 16-, 17- and 20-kDa band appears during the incubation of human osteosarcoma cell-conditioned medium, IGFBP-5 is not protected from proteolysis by the addition of either IGF-I or -II (Conover and Kiefer, 1993). In ovine and porcine small atretic follicles, IGFBP-5 proteolytic activity decreases significantly and in medium and large follicles IGFBP-5 proteolytic activity was lower in atretic versus healthy follicles (Besnard et al., 1996b; Monget et al., 1996; Besnard et al., 1997). In healthy growing follicles of ovine and porcine, there is an increase in proteolytic activity degrading IGFBP-5 (Besnard et al., 1996b; Besnard et al., 1997). In porcine, there was approximately 55% IGFBP-5 proteolytic activity in small, normal follicles and increased with follicle growth reaching more than 90% in preovulatory follicles, percentages are based on densitometric analysis of Western ligand blots (Besnard et al., 1997). In ovine follicular fluid, IGFBP-5 proteolysis is completely inhibited by EDTA and 1,10 phenanthroline and can be partially restored by the addition of calcium chloride and/or zinc chloride (Besnard et al., 1996b; Besnard et al., 1997). Collectively, these results indicate that ovine and porcine follicular fluid contains one or more metalloproteases that degrade IGFBP-2, -3, -4, and -5 (Besnard et al., 1996b; Besnard et al., 1997).

Protease activity for IGFBP-5 may be modulated by various hormones and growth factors. Cortisol may enhance IGFBP-5 protease activity in bone cells (Gabbitas et al., 1996), whereas FSH induces production of an IGFBP-5 protease in rat granulosa cells (Fielder et al., 1993; Liu et al., 1993). Proteolytic activity of IGFBP-5 is blocked by IGF-I or -II in granulosa cells (Fielder et al., 1993) and fibroblast-conditioned medium

(Camancho-Hubner et al., 1992). FSH may induce an IGFBP-5 protease, or it may decrease an IGFBP-5 protease inhibitor, which could lead to the activation of the IGFBP-5 protease.

There have been few investigations concerning a protease for IGFBP-6. In an investigation of the degradation of nonglycosylated and glycosylated IGFBP-6, glycosylated IGFBP-6 was shown to have a greater resistance to proteolysis than nonglycosylated IGFBP-6 (Neumann et al., 1998). Chymotrypsin was incubated with IGFBP-6, and was found to cleave intact nongylcosylated and glycosylated IGFBP-6 with 1 ng and 10 ng chymotrypsin, respectively (Neumann et al., 1998). Additional research is needed to further determine if IGFBP-6-specific proteolytic enzymes do in fact exist within the ovary.

In summary, recent studies have revealed intrafollicular proteases to be involved in degrading IGFBPs in follicular fluid. FSH has been shown to induce the production of a protease in rat granulosa cells that degrades IGFBP-4 and -5 (Liu et al., 1993). Besnard et al., (1996b) have shown follicular fluid of ovine preovulatory follicles to contain a metallo-dependent protease which can degrade IGFBP-4, -5 and lesser amounts of IGFBP-2. Gonadotropins in growing healthy follicles decrease the expression of IGFBPs <40-kD and increase secretion of protease(s) that degrade locally and peripheric IGFBPs <40-kD (Monget et al., 1996). In contrast, atretic follicles exhibit an increase in the expression of IGFBPs <40-kD. IGFBP -2, -3, -4 and -5 are proteolytically cleaved by porcine and ovine, IGFBP-2 and -5 in bovine, IGFBP-3 and -4 in women, and IGFBP-3 and -5 in rat follicular fluid. The protease(s) involved in the degradation of IGFBP-2, -3, -4 and -5 is thought to belong to the metalloprotease family and are calcium- and zinc-

dependent (Besnard et al., 1997). Hormones are believed to control the regulation of IGFBP-3; insulin may play a role in IGFBP-3 proteolysis (Bang et al., 1994). In porcine granulosa cells, the addition of IGF-I or-II diminishes the proteolysis of rhIGFBP-3, yet FSH, estradiol and GH had no significant effect (Grimes and Hammond et al., 1994). Insulin had no effect on IGFBP-4 proteolysis, however IGF-I increased the amount of IGFBP-4 fragments (Cohick et al., 1993). FSH and estradiol have been shown to induce IGFBP-4 proteolysis in women (Iwashita et al., 1996). The reduction of IGF-I levels may increase IGFBP-4 proteolysis in women (Cwyfan-Hughes et al., 1997). FSH induces the production of a protease for IGFBP-5 in rat granulosa cells (Fielder et al., 1993; Liu et al., 1993). Thus, it appears that proteolysis of IGFBPs and the hormonal regulation of proteolysis are species specific.

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CHAPTER III

CHANGES IN FOLLICULAR FLUID STEROIDS, INSULIN-LIKE GROWTH FACTORS (IGFs) and IGF BINDING PROTEINS (IGFBP) AND IGFBP PROTEOLYTIC ACTIVITY DURING EQUINE FOLLICULAR DEVELOPMENT.

ABSTRACT: Final growth of ovulatory follicles in the mare is characterized by increased steroidogenesis and changes in IGFBP. The objective of the present study was to evaluate changes in equine follicular fluid (FF) steroid, IGF and IGFBP content as well as IGFBP proteolytic activity during follicular development. Mares (n=14) were classified as either in the follicular phase (n = 8) or luteal phase (n = 6). Follicles (n=92) were categorized as small (< 16 mm), medium (16-25 mm), or large (> 25 mm), and follicular fluid was collected. Follicular fluid estradiol and androstenedione levels were greater ($P< 0.05$) and IGFBP-3 levels tended to be greater ($P < 0.10$) in large than in small or medium follicles, whereas IGFBP-2, -4 and -5 levels were less ($P< 0.05$) in large than in small or medium follicles. Estradiol and androstenedione concentrations were negatively correlated ($P< 0.01$) with IGFBP-2, -4, and -5 but not IGFBP-3 levels. To evaluate proteolysis of IGFBPs, FF was incubated with human ^{125}I-labeled IGFBP-2, -3, and -5 and protein separated by 12% SDS-PAGE. Follicular fluid did not cause proteolysis of ^{125}I-lableled IGFBP-2 or -3. However, ^{125}I-labeled IGFBP-5 was cleaved ($P< 0.05$) by FF from large follicles collected during the follicular phase, but not luteal

phase indicating that a protease to IGFBP-5 exists in estrogen dominant follicles. This IGFBP-5 protease was inhibited by kallikrein/serine and metalloprotease inhibitors. Little or no IGFBP-5 proteolytic activity was detected in FF from small or medium follicles from follicular and luteal phase mares. We conclude that the tendency of estrogen dominant follicles of mares to have greater levels of IGFBP-3 and lesser levels of IGFBP-2 do not appear to be due to differences in proteolysis, whereas changes in IGFBP-5 levels are likely due to changes in activity of a serine protease or metalloprotease. Changes in IGFBP may alter levels of bioavailable IGFs that stimulate steroidogenesis and mitogenesis in developing mare follicles.

Introduction

The mare is a seasonally polyestrous, long day breeder. During the breeding season, a mare's ovary will have multiple follicles of various sizes. One of these follicles will be selected to deviate and become a dominant, steroidogenically active follicle, while regression of the others occurs (Gastal et al., 1997). This dominant follicle will continue its growth, reaching ovulatory status with a diameter between 35 and 55 mm (Ginther and Pierson, 1989; Spicer et al., 1991). As follicular growth occurs, there is an increase in follicular fluid (FF) estradiol-17β (E_2) and androstenedione (A_4) levels (Kenney et al., 1979; Fay and Douglas, 1987; Spicer et al., 1991).

Insulin-like growth factor-I (IGF-I) increases steroidogenesis and mitogenesis of ovarian cells (for review, see Spicer and Echternkamp, 1995). In cattle, sheep, and swine, follicular growth and regression are associated with changes in insulin-like growth

factor binding protein (IGFBP) content more than with changes in IGF-I or -II (Spicer and Echternkamp, 1995; Stewart et al., 1996). IGFBPs are 22-45 kDa high affinity carrier proteins (Chan and Spencer, 1997) that bind IGF-I, prolong its half-life and block its biological action (for review see Clemmons, 1993; Hwa et al., 1999). Within the ovary, IGFBPs have the ability to inhibit the synergistic effects of IGF-I and FSH (Ui et al., 1989; Spicer et al., 1999) and may play a role in follicular atresia (Giudice, 1992). Follicular fluid levels of IGFBP-2, -4, and -5 decrease during follicular growth and increase during atresia in cattle, sheep, swine and humans (for review, see Spicer and Echternkamp, 1995; Monget et al., 1996), while levels of IGFBP-3 remain constant during follicular growth in cattle (Echternkamp et al., 1994a; Stewart et al., 1996) and humans (Cataldo and Giudice, 1992), but tend to increase slightly in sheep (Monget et al., 1993) and swine (Grimes et al., 1994). A recent study in horses indicates that growth of the dominant follicle is associated with a decrease in IGFBP-2 and IGFBP-5 whereas IGFPB-3 and -4 are unchanged (Gerard and Monget, 1998).

IGFBPs can be regulated through changes in gene transcription or proteolysis. One mechanism to enhance IGF-I's bioavailability to cells is through proteolysis of IGFBPs, particularly lower molecular weight IGFBPs (Besnard et al., 1996; Rajarm et al., 1997). During follicular growth in sheep, proteolytic activity for IGFBP-3 is decreased, whereas proteolytic activity for IGFBP-4 is greatly increased and IGFBP-2 and -5 is increased to a lesser extent (Besnard et al., 1996). In swine, proteolytic activity for IGFBP-2, -4, and -5 increases during follicular development and there is very little or no proteolytic activity for IGFBP-3 (Besnard et al., 1997). Currently, little information is available concerning the proteolytic activity during folliculogenesis of the mare. Therefore, the objective of

this study was to evaluate changes in equine follicular fluid (FF) steroid and IGFBP levels as well as IGFBP proteolysis during follicular steroidogenesis and development.

Materials and Methods

Biological Material

FF was aspirated from follicles (n=92) of 14 cyclic mares was collected at an abattoir and centrifuged (Langhout et al., 1991) and FF was stored at -20°C. The mares were classified as either in the follicular (n=8) or luteal phase (n=6) based on gross ovarian morphology; ovaries with a viable (vasculature visible) corpus luteum (CL) were classified as being in the luteal phase and ovaries with no CL, or with a corpus albicans were classified as being in the follicular phase. External diameters of follicles were measured to categorize them as being small (6-15 mm), medium (16-25 mm), or large (> 25 mm).

Radioimmunoassays (RIA)

Concentration of estradiol-17β in FF was determined by RIA as previously described (Spicer and Enright, 1991). The intra- and interassay coefficient of variance (CV) were 12.8 and 21.1%, respectively. Concentrations of androstenedione in FF were determined in a single assay using a commercial solid-phase RIA kit (ICN Biomedicals, Costa Mesa, CA) as previously described by Stewart et al. (1996). The intraassay CV was 15%. Concentrations of progesterone in FF were determined by RIA as previously described by Spicer and Enright (1991). The intra- and interassay CV were 13.3 and 6.7%, respectively. Concentrations of IGF-I in FF were determined by RIA as previously described (Echternkamp et al., 1990; Spicer et al., 1991). The intra- and interassay CV

were 14.5 and 15.5%, respectively. Concentrations of IGF-II in FF were determined by RIA in a single assay as previously described Spicer et al. (1995). The intraassay CV was 3.2 %.

Ligand Blots

IGF binding proteins (IGFBP) in FF were analyzed by one-dimensional SDS-PAGE, as previously described (Echternkamp et al., 1994a; Simpson et al., 1997). Briefly, 4 μl of FF was mixed with 21 μl of denaturing buffer (BIORAD, Hercules, CA). The samples were heat denatured (3 min at 100°C), subsequently centrifuged at 4657 X g for 3 min and separated using 12% PAGE for about 18 to 20 h, at a constant current and varying voltage. After separation, proteins in the gels were electrophoretically transferred to nitrocellulose paper (Midwest Scientific, St. Louis, MO) for 2.5 to 3.0 h and ligand-blotted overnight with ^{125}I-IGF-II. Tris-buffered saline (TBS) with 0.1% Tween was used to wash the nitrocellulose blots. The nitrocellulose blots were dried and exposed to X-ray film at -70°C for 12 d. X-ray film was then developed and band intensity on autoradiographs was determined using scanning densitometry with a Molecular Analyst (BIORAD, Hercules, CA).

IGFBP Iodination

IGFBP-2, -3, and -5 were iodinated by a modification of the chloramine-T method. Recombinant human IGFBP-2 (31,000 molecular weight) was obtained from Austral Biologicals (San Ramon, CA); recombinant human IGFBP-3 (47,000 molecular weight) and recombinant human IGFBP-5 (30,000 molecular weight) were obtained from Upstate

Biotechnology Inc. (Lake Placid, NY). Two micrograms of IGFBP-2, -3, and -5 were independently incubated with 1 mCi Na [^{125}I] in the presence of 3.2 μg chloramine-T for 30 s at 25°C. The reaction was stopped with addition of 6μg sodium meta-bisulfate. ^{125}I-IGFBP-2, -3, and -5 was purified from the reaction mixture by Sephadex G-50 gel chromatography (Sigma Chemical Co., St. Louis, Mo, USA).

IGFBP Proteolysis

A subset of 36 of 92 follicles were selected for IGFBP proteolysis assay based on: 1) the follicle with the greatest E_2 concentration was selected from each mare, and 2) one small and one medium follicle, if present, was randomly selected from each mare. FF (6 μl) was incubated at 37°C for 5 h with 60,000 cpm of either recombinant human ^{125}I-labeled IGFBP-2, -3, or -5. Proteolytic fragments of the various ^{125}I-labeled IGFBPs were separated by 12% SDS-PAGE. Prior to electrophoresis, samples were boiled for 3 min to denature proteins and then added to the wells in the gel. Gels were dried on a vacuum and exposed to X-ray film for 48 h. The resultant breakdown products or lack of were determined using scanning densitometry with a Molecular Analyist (BIORAD, Hercules, CA.).

Protease Inhibitors to IGFBP Degradation

Inhibition of FF protease activity to IGFBP was evaluated using 60,000 cpm of recombinant human ^{125}I-labeled IGFBP-5 in media with 0.01 or 0.10 mM aprotinin (a kallikrein and serine protease inhibitor), 0.10 or 1.0 mM of either phenylmethane-sulfonyl fluoride (PMSF) (a serine, cysteine, and thiolprotease inhibitor) (Boehringer Mannheim, Indianapolis, IN), 1,10-phenanthroline (metalloprotease inhibitor) or doxycycline (metalloprotease inhibitor), 0.001, 0.01 or 0.10 mM cyclohexylacetyl-phe-

arg-ser-val-gln amide (CAA) (kallikrein protease inhibitor) or 0.10, 1.0 or 5.0 mM EDTA (chelates Ca^{++}) (Sigma Chemical Co., St. Louis, MO), 6 μl of FF were added to each tube and incubated at 37°C for 5 h. Samples were separated by 12% SDS-PAGE, subjected to autoradiography, and resultant bands scanned as previously described.

Statistical analyses

Follicular fluid hormone and IGFBP data as well as proteolysis data were analyzed using least squares ANOVA by the General Linear Models procedure of SAS (1988). Main effects, consisting of follicle size (small, medium, large) and follicle phase (follicular, luteal) and their various interactions were analyzed. Means were compared using "t"-test if significant main effects were observed. Relationships among variables measured were evaluated by simple correlation analysis (Pearson correlation coefficients) (SAS, 1988). Means are presented as least squares means ± SE.

Results

A significant ($P < 0.0001$) main effect of follicle size, but not phase or size x phase interaction ($P > 0.10$) was observed with follicle diameter. Diameter averaged 33 ± 1.0 mm, 17.8 ± 0.70 mm and 11.4 ± 0.48 mm, for large, medium and small follicles, respectively.

Follicle size, phase of estrous cycle or their interaction did not affect ($P > 0.10$) FF progesterone levels. FF progesterone averaged 33.4 ± 8.1 ng/ml, 23.8 ± 5.7 ng/ml and 30.7 ± 3.5 ng/ml, for small, medium and large follicles, respectively.

Follicle size x phase interaction affected ($P < 0.005$) FF estradiol levels (Figure 1). Main effects of follicle size and phase were also significant ($P < 0.005$). Large, follicular phase follicles (1425.1 ± 114.2 ng/mL) contained greater ($P < 0.0001$) levels of estradiol than large follicles in the luteal phase (473.3 ± 228.4 ng/mL), small follicles in the follicular (46.3 ± 77.6 ng/mL) or luteal phase (19.9 ± 74.8 ng/mL), and medium follicles in either the follicular (41.2 ± 98.9 ng/mL) or luteal phase (84.6 ± 149.5 ng/mL).

A significant ($P < 0.0005$) main effect of follicle size, but not phase or size x phase interaction was observed for FF androstenedione levels. Large follicles had 6- to 8-fold greater ($P < 0.05$) concentrations of androstenedione than small or medium follicles (Figure 2A).

Follicle size affected ($P < 0.01$) FF IGF-I levels, whereas phase of estrous cycle and size x phase had no effect on FF IGF-I levels. Large follicles contained 78% to 90% more ($P < 0.05$) IGF-I than did small or medium follicles (Figure 2B).

Phase of estrous cycle tended to affect ($P < 0.10$), whereas size and size x phase had no significant effect on FF IGF-II levels. Follicular phase follicles (67.7 ± 3.0 ng/mL) tended to have greater ($P < 0.10$) amounts of IGF-II than did luteal phase follicles (57.0 ± 5.0 ng/mL).

Follicle size and phase ($P < 0.05$), but not size x phase interaction affected concentrations of protein in FF. FF of large follicles (145.7 ± 8.5 mg/mL) had 17% to 47% greater ($P < 0.05$) concentrations of protein than small (100.6 ± 4.2 mg/mL) and medium (124.7 ± 5.9 mg/mL) follicles. There were greater ($P < 0.05$) concentrations of protein in FF of luteal phase (131.5 ± 6.3 mg/mL) than follicular phase (115.8 ± 3.9 mg/mL) follicles.

Ligand blotting with [^{125}I] IGF-II revealed at least six forms of IGFBP present in FF: 125-135-kDa IGFBP, 115-kDa, 90-96-kDa, 40- to 44-kDa (IGFBP-3), 34-kDa (IGFBP-2), 27-29-kDa (IGFBP-5), and 20-22-kDa (IGFBP-4) (Figure 3). Follicle size ($P < 0.01$) but not phase of cycle or size x phase interaction ($P > 0.10$) affected IGFBP-2 levels in FF. Concentrations of IGFBP-2 in small and medium follicles were 1.8- and 1.9-fold greater ($P < 0.05$) than in large follicles (Figure 2C). There tended ($P < 0.10$) to be an interaction between follicle size x phase on FF IGFBP-3 levels (Figure 4A). Large luteal phase follicles had 60% greater ($P < 0.05$) IGFBP-3 concentrations than did large follicular phase follicles, and had 48%, 57%, and 60% greater ($P < 0.05$) concentrations of IGFBP-3 than medium luteal, small follicular, and small luteal phase follicles, respectively.

Follicle size x phase interaction affected ($P < 0.05$) IGFBP-4 levels in FF (Figure 4B). Concentrations of IGFBP-4 were 3.0- and 2.6-fold greater ($P < 0.05$) in small follicular phase and small luteal phase follicles than in both large follicular phase and medium luteal phase follicles, respectively. Medium follicular phase follicles had 3.3-fold greater ($P < 0.05$) concentrations of IGFBP-4 than both medium luteal phase and large follicular phase follicles.

Follicle size x phase interaction affected ($P < 0.05$) IGFBP-5 levels in FF (Figure 4C). Main effect of follicle size was also significant ($P < 0.005$). Concentrations of IGFBP-5 in medium and small follicular phase follicles, and small luteal phase follicles were 5-, 4.1-, and 4-fold greater ($P < 0.05$) than in large follicular phase follicles, respectively. Concentrations of IGFBP-5 were 2.3- and 2.1-fold greater ($P < 0.05$) in medium

follicular phase follicles than large luteal phase and medium luteal phase follicles, respectively.

Follicle size tended ($P < 0.10$) to affect levels of the 115-kDa IGFBP in FF. Small (0.69 ± 0.09 ADU) and medium (0.81 ± 0.14 ADU) follicles had 3.1- and 3.6-fold greater ($P < 0.05$) concentrations of the 115-kDa IGFBP, respectively, than did large (0.23 ± 0.20 ADU) follicles. The 135-kDa and 125-kDa IGFBP and were arithmetically combined for analysis and labeled as 125-135-kDa IGFBP. Follicle size x phase interaction affected ($P < 0.05$) 125-135-kDa IGFBP levels in FF. Main effects of follicle size were also significant ($P < 0.05$). Medium follicular phase follicles (2.6 ± 0.26 ADU) had 1.5- and 1.6-fold greater ($P < 0.05$) concentrations of 125-135-kDa IGFBP than did large follicular phase (1.7 ± 0.32 ADU) and small follicular phase follicles (1.6 ± 0.23 ADU), respectively. Medium follicular and luteal phase follicles (2.1 ± 0.39 ADU) as well as small luteal phase follicles (2.2 ± 0.22 ADU) had 3- to 4-fold greater ($P < 0.05$) concentrations of 125-135-kDa IGFBP than did large luteal phase follicles (0.6 ± 0.61 ADU). Large follicular phase (3.9 ± 1.3 ADU) versus large luteal phase (0.58 ± 2.5 ADU) follicles did not differ ($P > 0.10$).

The 96-kDa and 90-kDa IGFBP were combined for analysis and labeled as 90-96-kDa IGFBP. Follicle size x phase interaction tended to affect ($P < 0.10$) the 90-96-kDa IGFBP levels in FF. Medium follicular phase follicles (11.9 ± 2.4 ADU) had 3.2- and 2.6-fold greater ($P < 0.05$) concentration of 90-96-kDa IGFBP than large luteal phase follicles (1.3 ± 5.5 ADU) and small follicular phase follicles (2.9 ± 2.1 ADU), respectively. Medium luteal phase follicles (10.2 ± 3.6 ADU) had 2.6-fold greater ($P < 0.05$) concentrations of the 90-96-kDa IGFBP than did small follicular phase follicles

(2.9 ± 2.1 ADU). Small luteal phase follicles (7.8 ± 2.0 ADU) had 2.1-fold greater ($P < 0.05$) concentrations of the 90-96-kDa IGFBP than did small follicular phase follicles (2.9 ± 2.1 ADU). Large follicular phase (7.1 ± 2.9 ADU) versus large luteal phase (1.3 ± 5.5 ADU) follicles did not differ ($P > 0.10$).

FF caused little or no proteolysis ($P > 0.10$) of ^{125}I-labeled IGFBP-2 or -3 (Figure 5). Amount of ^{125}I-labeled IGFBP-2 degradation (large, 24.7 ± 1.4 %; medium, 24.9 ± 1.5 %; and small, 21.8 ± 0.98 %) after 5 h incubation was not affected ($P < 0.10$) by follicle size, phase of cycle or follicle size x phase interaction. Similarly, ^{125}I-labeled IGFBP-3 degradation (large 50.2 ± 3.2 %; medium, 48.5 ± 3.4 %; and small, 45.9 ± 2.3 %)was not affected ($P < 0.10$) by follicle size, phase of cycle or follicle size x phase interaction. However, ^{125}I-labeled IGFBP-5 was cleaved more by FF from large follicles collected during the follicular phase than the luteal phase (Figure 6). Intact IGFBP-5 migrated at 30-kDa, and breakdown products at approximately 19-kDa, 16-kDa, and 14.5-kDa (Figure 6). A significant ($P < 0.05$) interaction between follicle size and phase of estrous cycle was observed for the 30-kDa intact band with lesser amounts ($P < 0.005$) of the 30-kDa band found after incubation with FF from large follicular phase follicles (68.2 ± 4.7 ADU) than from large luteal phase follicles (92.9 ± 7.6 ADU), medium follicular (86.8 ± 5.9 ADU) or luteal (90.3 ± 7.6 ADU), or small follicular (84.8 ± 3.9 ADU) or luteal (85.1 ± 4.9 ADU) phase follicles.

A significant ($P < 0.05$) interaction was also observed between follicle size and phase on the 19-kDa band, the most prevalent breakdown product of ^{125}I-IGFBP-5. Large follicular phase follicles had 2.5- to 4.3-fold greater ($P < 0.05$) amounts of the breakdown

band than did all other follicle classes (Figure 7A). Follicle size and phase of estrous cycle affected ($P < 0.05$) the 19-kDa breakdown product as well.

There tended ($P < 0.10$) to be an interaction between follicle size and phase on the 16-kDa breakdown product of ^{125}I-IGFBP-5 (Figure 7B). Large follicular phase follicles had 2.0- to 3.3-fold greater ($P < 0.05$) amounts of the 16-kDa band than did all other follicle classes. Follicle size and phase of estrous cycle affected ($P < 0.05$) the 16-kDa breakdown product. Large follicles (6.1 ± 1.0 ADU) had greater amounts ($P < 0.05$) of 16-kDa band than did small (2.9 ± 0.8 ADU), but not medium follicles (3.8 ± 1.1 ADU).

There tended ($P < 0.10$) to be an interaction between follicle size and phase on the 14.5-kDa breakdown product of ^{125}I-IGFBP-5 (Figure 7C). Large follicular phase follicles had 2.2- to 3.0- fold greater ($P < 0.05$) amounts of 14.5-kDa breakdown product than did all other follicle classes. Follicle size and phase of the estrous cycle also tended to affect ($P < 0.10$) the 14.5 kDa breakdown product.

Serine protease inhibitors CAA and aprotinin inhibited ($P < 0.01$) IGFBP-5 protease activity (% of control) (Figure 8A). CAA at 0.01 mM and 0.10 mM caused greater ($P < 0.01$) inhibition of IGFBP-5 protease activity than at 0.001 mM (Figure 8A). PMSF at 1.0 mM had little or no effect on IGFBP-5 protease activity (Figure 8A). Aprotinin inhibited IGFBP-5 protease activity to an extent similar to that of CAA; the inhibition caused by 0.01 mM and 0.10 mM of aprotinin did not differ ($P > 0.10$) (Figure 8A).

The metalloprotease inhibitors doxycycline, EDTA, and 1,10-phenanthroline affected ($P < 0.01$) IGFBP-5 protease activity (% of control) (Figure 8B). Doxycycline at 1.0 mM caused greater ($P < 0.01$) inhibition of IGFBP-5 protease activity than at 0.10 mM (Figure 8B). Only 5.0 mM EDTA inhibited ($P < 0.01$) IGFBP-5 protease activity (Figure

8B). 1,10-Phenanthroline caused greater ($P < 0.01$) inhibition of IGFBP-5 protease activity at 1.0 mM than at 0.10 mM (Figure 8B).

Follicular fluid E_2, A_4, IGF-I and IGF-II concentrations were negatively correlated ($P < 0.05$) with levels of IGFBP-2 and -5 but not with ($P > 0.10$) IGFBP-3 levels (Table 1). FF IGFBP-4 was negatively correlated ($P < 0.05$) with only FF E_2 and IGF-I. Follicular fluid P_4 concentrations were negatively correlated ($P < 0.05$) with IGFBP-3 and IGFBP-5 but not correlated with IGFBP-2 or -4 (Table 1). Follicle size (diameter) was negatively correlated ($P < 0.05$) with IGFBP-2, IGFBP-4, IGFBP-5 and positively correlated ($P < 0.05$) with E_2 ($r = 0.73$), A_4 ($r = 0.52$), and IGF-I ($r = 0.49$). Concentrations of E_2 in FF were positively correlated with IGF-I ($r = 0.49$) ($P < 0.0001$) and IGF-II ($r = 0.35$) ($P < 0.01$).

Amount of IGFBP-5 proteolysis was positively correlated ($P < 0.01$) with FF E_2 levels and to a lesser extent ($P < 0.05$) FF A_4 levels (Table 2). ^{125}I-IGFBP-5 fragments were negatively correlated ($P < 0.01$) with IGFBP-2 and -5 but not with IGFBP-3 or -4 (Table 2). Follicular fluid IGF-I and -II concentrations were positively correlated ($P < 0.05$) with ^{125}I-IGFBP-5 fragments (Table 2).

Discussion

Results of the present study revealed that: 1) ^{125}I-labeled IGFBP-5 was cleaved by mare FF from large follicles collected during the follicular phase, but not the luteal phase, whereas FF did not cause proteolysis of ^{125}I-labeled IGFBP-2 or -3; 2) the amount of IGFBP-5 proteolysis was positively correlated with follicular fluid E_2, A_4, IGF-I and IGF-II levels; 3) follicular fluid IGFBP-2, -4, -5 and 125-135-kDa IGFBP were less in large than in small or medium follicles, particularly during the follicular phase; 4)

follicular fluid IGF-I levels were greater in large than in small or medium follicles, whereas FF IGF-II levels tended to be greater in follicular phase vs. luteal phase follicles; and 5) metalloprotease and serine protease inhibitors significantly reduced IGFBP-5 protease activity; in a dose dependent fashion; and 6) follicular fluid E_2 and A_4 levels were greater in large than in small or medium follicles and follicular phase mares had greater E_2 in large follicles vs. luteal phase mares.

For the first time, proteolysis of IGFBP-5 in mare FF has been evaluated. In particular, we found evidence for IGFBP-5 proteolysis, but none for IGFBP-2 or -3. Similar to the present study, evidence for proteolysis of IGFBP-5 has been found in bovine (Stanko et al., 1994; Spicer and Chamberlain, 2000), ovine (Besnard et al., 1996; Monget et al., 1996) and porcine (Besnard et al., 1997) FF. Similar to results found in other mammalian species (for review, see Spicer and Echternkamp, 1995; Monget et al., 1996), we observed IGFBP-5 levels to be less in large than in small or medium follicles. In particular, IGFBP-5 levels in FF of subordinate large or small follicles from cattle are severalfold greater than in dominant follicles (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996). Also in agreement with the present study, intrafollicular IGFBP-5 levels were greater in large nonovulatory and subordinate/atretic follicles of mares (Gerard and Monget, 1998). FSH has been reported to regulate IGFBP production by rat granulosa cells (Adashi et al., 1990; 1991). Thus, changes in intraovarian levels of IGFBP-5 may be regulated by changes in local synthesis as well as proteolysis of IGFBP-5. Proteolysis of ^{125}I-IGFBP-5 was positively correlated with FF E_2 ($r = 0.5$-0.7) and A_4 ($r = 0.2$-0.4) levels in the present study. This latter observation coupled with the observation that FF levels of IGFBP-5 are negatively correlated with E_2 and A_4 levels,

implies either or both steroids may regulate IGFBP-5 levels via induction of protease activity. Fielder et al., (1993) and Liu et al., (1993), found FSH to induce an IGFBP-5 protease in rat granulosa cells *in vitro*, and this protease was inhibited by IGF-I or IGF-II. Similarly, IGF-I activates IGFBP-4 protease in cultured rat granulose cells (Liu et al., 1993). Because IGFBP-5 protease was positively correlated ($r = 0.4$ to 0.5) with FF levels of IGF-I and -II, it is unlikely that IGF-I or -II inhibits this protease activity in mare follicles. In support of this latter suggestion, IGFBP-5 proteolysis was not altered by IGF-I or -II in osteocarconoma cells (Conover and Keifer, 1993). Whether IGF-I and -II directly activate IGFBP-5 protease activity in mare follicles or whether the effects are indirect via IGF-I induction of steroidogenesis (Spicer and Echternkamp, 1995) will require further study.

Consistent with the present study, others have reported IGFBP-2 levels are greater in small than in medium or large follicles in sheep (Spicer et al., 1995), pigs (Mondschein et al., 1991; Echternkamp et al., 1994b) and cattle (Echternkamp et al., 1994a; Stewart et al., 1996). Follicular fluid of dominant follicles of cattle (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996) and mares (Gerard and Monget, 1998) contain less IGFBP-2 than FF of subordinate follicles. Atretic follicles contain more IGFBP-2 than do healthy follicles in sheep (Monget et al., 1993), cattle (Echternkamp et al., 1994a), and women (San Roman and Magoffin, 1993). Proteolysis of IGFBP-2 by FF has been reported for cattle (Stanko et al., 1994), sheep (Besnard et al., 1996), and pigs (Besnard et al., 1997). We found no evidence for FF proteolysis of IGFBP-2 in mare follicles, and thus changes in IGFBP-2 levels are likely a result of changes in local production of IGFBP-2 within mare follicles. This later notion awaits verification. Because FF levels

of E_2, A_4, IGF-I and IGF-II were negatively correlated with FF IGFBP-2 levels in the present study, further research should focus on the role these hormones play in regulating follicular IGFBP-2 production in the mare.

Similar to the present study, IGFBP-3 was found to be the major IGF binding protein in FF of weaned sows (Howard and Ford, 1992), humans (Cataldo and Giudice, 1992; San Roman and Magoffin, 1993), and cattle (Echternkamp et al., 1994; Funston et al., 1996; Stewart et al., 1996). In ewes, IGFBP-3 levels increase during follicular growth in some (Monget et al., 1993) but not other (Spicer et al., 1995) studies. Similarly in pigs, IGFBP-3 content increase during follicular growth in some (Grimes et al., 1994) but not other (Echternkamp et al., 1994) studies. A recent study in mares indicates that FF IGFBP-3 levels do not vary with physiological status of the follicle (Gerard and Monget, 1998). In contrast, we found FF IGFBP-3 levels tended to be greater in large luteal phase follicles when compared to large follicular phase follicles, but were not different among any other classes of follicles. Reasons for the discrepancies among studies are unknown but based on the present results IGFBP-3 levels may be influenced by stage of cycle when follicles are collected. In contrast to studies in women (Giudice et al., 1990; Hossenlopp et al., 1990; Davies et al., 1991), pigs (Besnard et al., 1997), sheep (Besnard et al., 1996) and rats (Davenport et al., 1990), we found no evidence for IGFBP-3 protease activity in FF. Whether these differences are due to species differences or due to some other factor is unclear. Because no proteolysis of IGFBP-3 was detected in the present study, the changes in FF IGFBP-3 observed in the mare are likely due to factors other than proteolysis such as changes in IGFBP-3 transudation from serum as suggested for cattle (Echternkamp et al., 1994).

In pigs (Howard and Ford, 1992), humans (Cataldo and Giudice, 1992), and cattle (Echternkamp et al., 1994a; Stewart et al., 1996), FF IGFBP-4 levels are undetectable in estrogen-active and dominant follicles. In atretic and late atretic follicles of sheep, the 22- to 24-kDa band (IGFBP-4) was found to be intense (Monget et al., 1993), while in estrogen-active preovulatory ewe follicles the 22- to 24-kDa band was undetectable using Western ligand blotting (Monget et al., 1993; Spicer et al., 1995). Although not evaluated in the present study, protease activity for IGFBP-4 has been reported in dominant estrogenic follicles of women (Chandraskher et al., 1995), mare (Mazerbourg et al., 2000), bovine (Mazerbourg et al., 2000; Spicer and Chamberlain, 2000), porcine (Besnard et al., 1997; Mazerbourg et al., 2000) and ovine (Besnard et al., 1996; Mazerbourg et al., 1999) FF. Experiments using in situ hybridization have shown atresia to be characterized by an increase in IGFBP-4 gene expression in rat granulosa cells (Nakatani et al., 1991). Thus, as with IGFBP-2 and -5, intraovarian changes in IGFBP-4 levels may be regulated by changes in local synthesis as well as proteolysis of IGFBP-4. We found large follicular phase follicles and medium luteal phase follicles to have less IGFBP-4 concentrations than any other class of follicles. Because absolute levels of IGFBP-4 were minor compared with all other IGFBPs in mare FF, and Gerard and Monget (1998) showed little or no change in IGFBP-4 levels during growth of the dominant follicle in mares, we chose not to evaluate its proteolysis by FF. Whether these minor changes in FF IGFBP-4 levels found in the present study are due to changes in local synthesis or proteolysis will require further study.

Similar to a 96-kDa IGFBP found in equine serum (Prosser and McLaren, 1992) and bovine serum (Simpson et al., 1997), we found a 90-to 96-kDa IGFBP in mare FF. As

previous studies have suggested, this 90-to 96-kDa IGFBP is most likely a proteolytic fragment of the soluble form of the IGF-II receptor (Westlund et al., 1991). We also found at least two other high-molecular weight IGFBPs in mare FF, a 115-kDa and a 125-135-kDa IGFBP. The 115- and 125-135-kDa IGFBPs are likely soluble forms of the IGF type I receptor. Interestingly, all of these high molecular weight IGFBP changed with follicular status, indicating that changes in IGF type I and II receptors may play a role in follicular development in the mare.

For the first time, protease inhibitors to IGFBP-5 proteolysis in mare FF has been evaluated. We found metalloprotease inhibitors such as doxycycline, EDTA, and 1,10-phenanthroline, as well as serine protease inhibitors CAA, PMSF, and aprotinin inhibited, in a dose-dependent way, IGFBP-5 protease activity, suggesting that the IGFBP-5 protease present in mare FF is a serine-metalloprotease. The IGFBP protease(s) that degrades IGFBP-2, -4 and -5 found in swine follicles was inhibited by metalloprotease inhibitors but not serine protease inhibitors (Besnard et al., 1997). However, a serine protease has been found to inhibit the degradation of IGFBP-3 in swine follicles (Grimes and Hammond, 1994). The IGFBP protease that degrades IGFBP-3, -4 and -5 found in sheep FF is a metallo-protease (Besnard et al., 1996). In humans, the IGFBP protease that degrades IGFBP-4 is a metallo-serine protease (Chandrasekher et al., 1995).

Estradiol is the dominant steroid secreted by the preovulatory follicle of the mare at the beginning of estrus (Kenney et al., 1979; Meinecke et al., 1987; Spicer et al., 1991). As ovulation approaches, the follicular content of androstenedione (Meinecke et al., 1987), testosterone (Watson and Hinrichs, 1988; Spicer et al., 1991) and estradiol (Spicer

et al., 1991) increases. At 3 cm in diameter, there appears to be a turning point in the maturation of equine follicles, in that > 3 cm healthy follicles have 3- to 5-fold greater levels of estrogens and androgens than healthy < 3 cm follicles (Kenney et al., 1979; Belin et al., 2000). In agreement with these and other studies in mares (Kenney et al., 1979; Fay and Douglas, 1987; Spicer et al., 1991), we found large (>25 mm) follicles in the follicular phase to have 3-fold greater concentrations of E_2 than large follicles in the luteal phase, and 17- to 72-fold greater amounts of estradiol than small or medium follicles in either the follicular or luteal phase. In agreement with studies in cattle (Kruip et al., 1979; Henderson and Franchimont, 1981; Ireland and Roche, 1982), size of antral follicles was positively correlated with FF estradiol and androstenedione concentrations. Increased FF E_2 and A_4 levels with follicular size suggest that these steroids are involved in follicular growth and development in the mare. Consistent with this suggestion, we found that FF E_2 and A_4 levels were negatively correlated with levels of IGFBP-2, -4, and -5 but not IGFBP-3 in FF. Previous studies have also reported negative correlation between FF estradiol and low molecular weight IGFBP levels in cattle (Echternkamp et al., 1994a; Stewart et al., 1996), sheep (Spicer et al., 1995), and mares (Gerard and Monget, 1998). Whether E_2 and A_4 regulate IGFBP levels via changes in local production or changes in proteolysis will require further study.

Follicular fluid IGF-I and -II concentrations in dominant and subordinant follicles did not differ in cattle (Stewart et al., 1996), yet FF concentrations of IGF-I increase (Spicer et al., 1988, 1992; Echternkamp et al., 1990; Spicer and Enright, 1991) and IGF-II decrease (Spicer and Echternkamp, 1995; Stewart et al., 1996) with follicle size in cattle. Similarly, we found FF IGF-I levels to be greater in large than in small or medium

follicles. However, FF IGF-II levels were not different among small, medium and large follicles but tended to be greater in follicular phase vs. luteal phase follicles. Whether these changes in FF IGF-I and -II are due to changes in local production of these IGFs will require further study.

In conclusion, follicular fluid E_2, A_4, and IGF-I levels increase in large follicles coincident with a reduction in IGFBP-2, -4 and -5 levels and an increase in proteolysis of IGFBP-5. Thus, increases in follicular fluid E_2, A_4 and IGF-I levels during the estrous cycle of the mare may modulate development of follicles and/or atresia by affecting the synthesis or proteolysis of IGFBPs.

Implications

Follicular growth, maturation, and atresia are IGF-dependent mechanisms that are affected by IGFBPs. Lower molecular weight IGFBPs were found to decrease in large, estrogenic follicles with increased E_2 and IGF-I levels. We were the first to report that proteolysis of IGFBP-5 by FF of the mare, and that proteolysis of IGFBP-2 and -3 is not present in mare FF. There is a need to focus future research on identifying the specific hormonal regulators of IGFBP-5 protease activity, as well as evaluate if IGFBP protease inhibitors exist in mare FF.

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No. 3), Monsanto Co. (St. Louis, MO) for supplying IGF-II, and Beltex Corporation (Ft. Worth, TX) for their generous donations of equine ovaries.

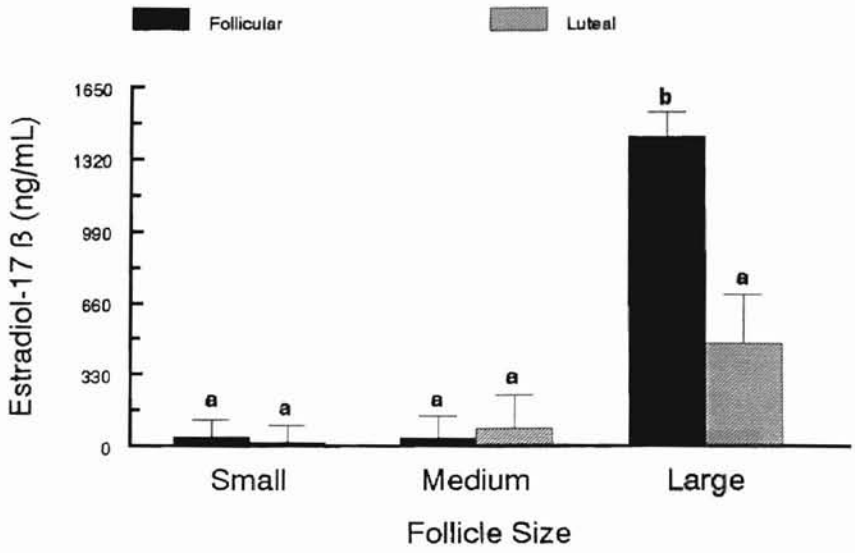


Figure 1. Follicular fluid concentrations of estradiol-17β collected from small (6-15 mm), medium (16-25 mm) or large (> 25 mm) follicles in either the follicular phase or luteal phase in mares. [a,b]Means without a common superscript differ ($P < 0.05$).

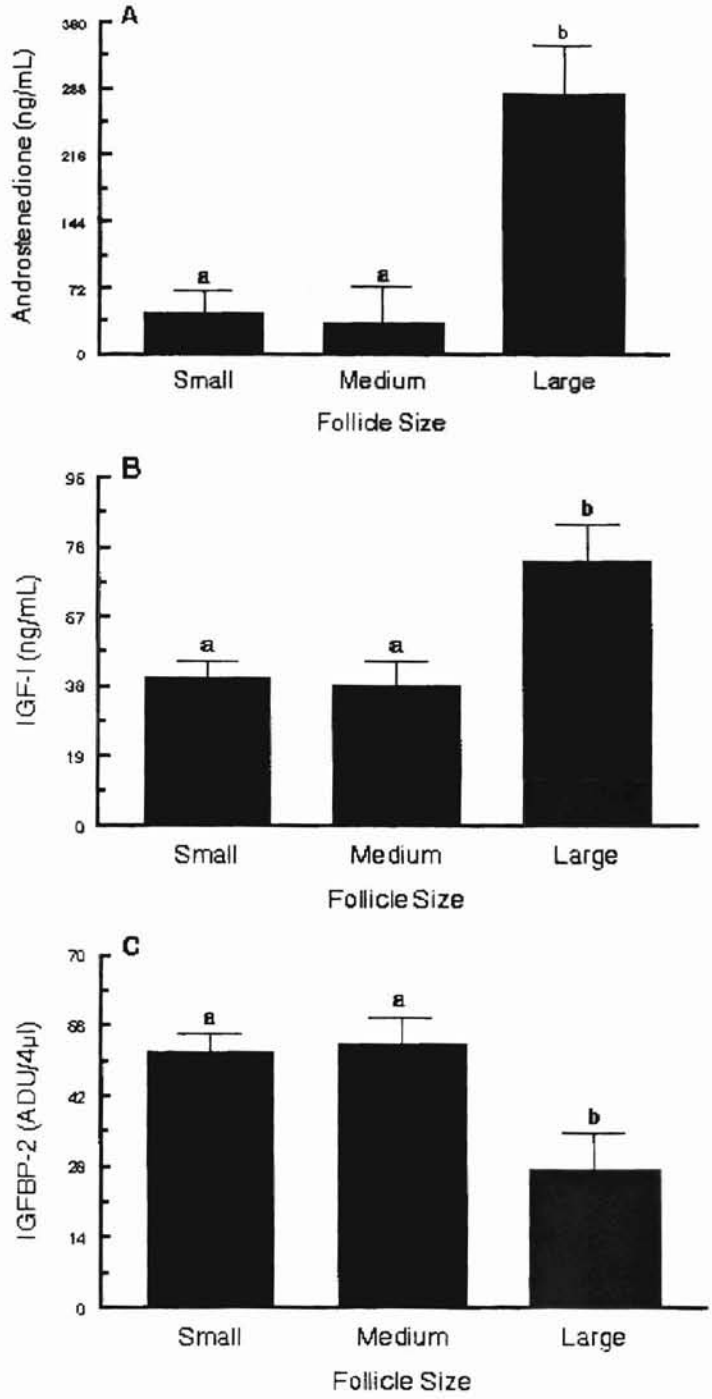
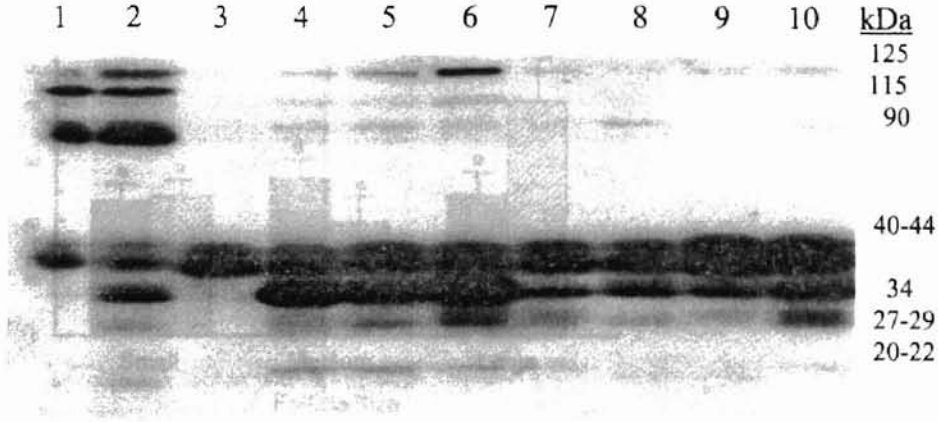


Figure 2. Follicular fluid concentrations of androstenedione (Panel A), IGF-I (Panel B), and IGFBP-2 (Panel C) collected from small (6-15 mm), medium (16-25 mm) or large (> 25 mm) follicles in either the follicular or luteal phase in mares. [a,b]Within a panel, means without a common superscript differ ($P < 0.05$).



Mare Number	1	1	2	2	2	2	2	2	3	3	
Follicle Size	36	17	44	18	17	11	6	33	30	8	(mm)
Estradiol-17β	2164	9	3225	8	8	1	31	20	1231	14	(ng/ml)

Figure 3. Representative ligand blot of IGFBPs in follicular fluid (FF) of mares. *Follicular fluid samples* (4 μl) from individual mares were evaluated for binding to ^{125}I-IGF-II as described in Materials and Methods. Lane 1-2: FF samples from large and medium follicles of mare 1 during the follicular phase; Lane 3-8: FF samples from large, medium and small follicles of mare 2 during the luteal phase; Lane 9-10: FF samples from large and small follicles of mare 3 during the luteal phase. Note the loss of IGFBP-2 and -5 in mare 1 and 2 with estrogenic follicles.

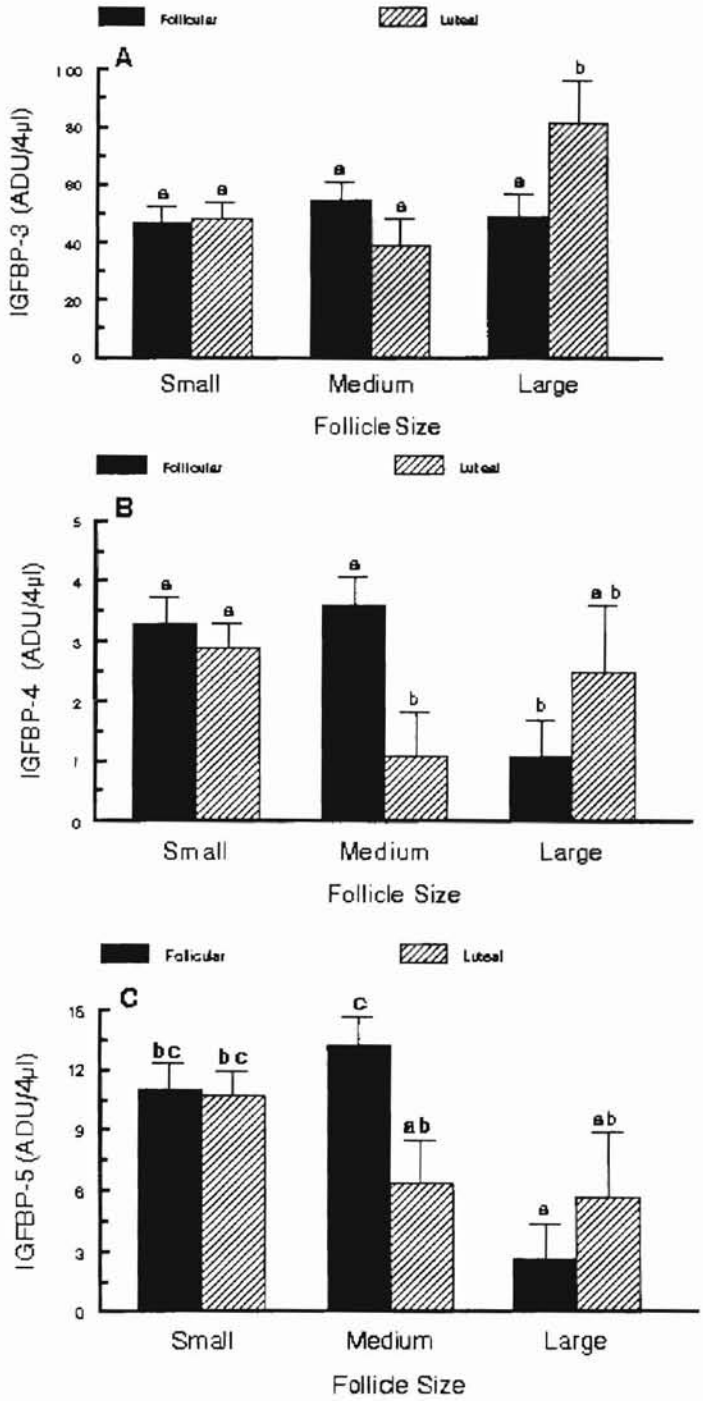
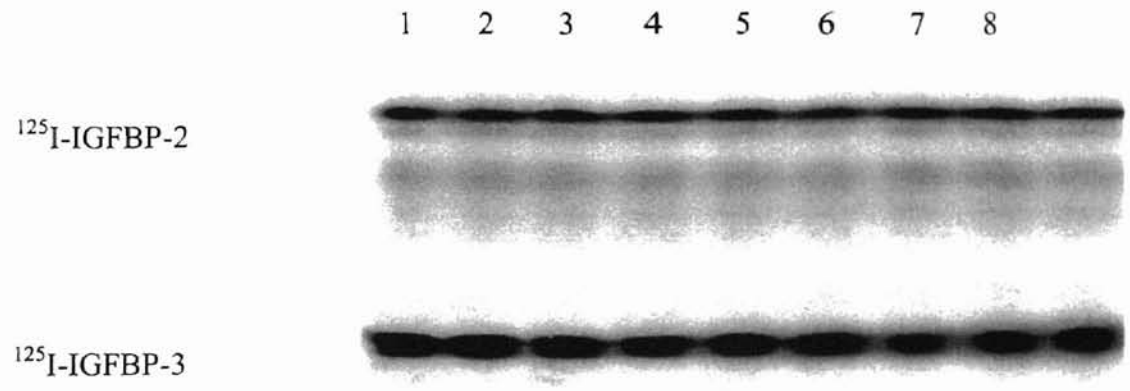


Figure 4. Binding activity of IGFBP-3 (Panel A), IGFBP-4 (Panel B), and IGFBP-5 (Panel C) in mare follicular FF collected from small (6-15 mm), medium (6-25 mm) or large (> 25 mm) follicles. [a,b,c]Within a panel, means without a common superscript differ ($P < 0.05$).



Mare Number	8	8	8	9	9	10	10	4
Ovarian Status	F	F	F	L	L	L	L	L
Follicle Size (mm)	30	20	10	18	9	9	23	35
Estradiol-17β (ng/ml)	1717	15	32	276	1	2	115	63

Figure 5. Representative autoradiograph measuring proteolytic activity of mare follicular fluid to recombinant human ^{125}I-labeled IGFBP-2 and -3. (F=follicular phase, L=luteal phase).

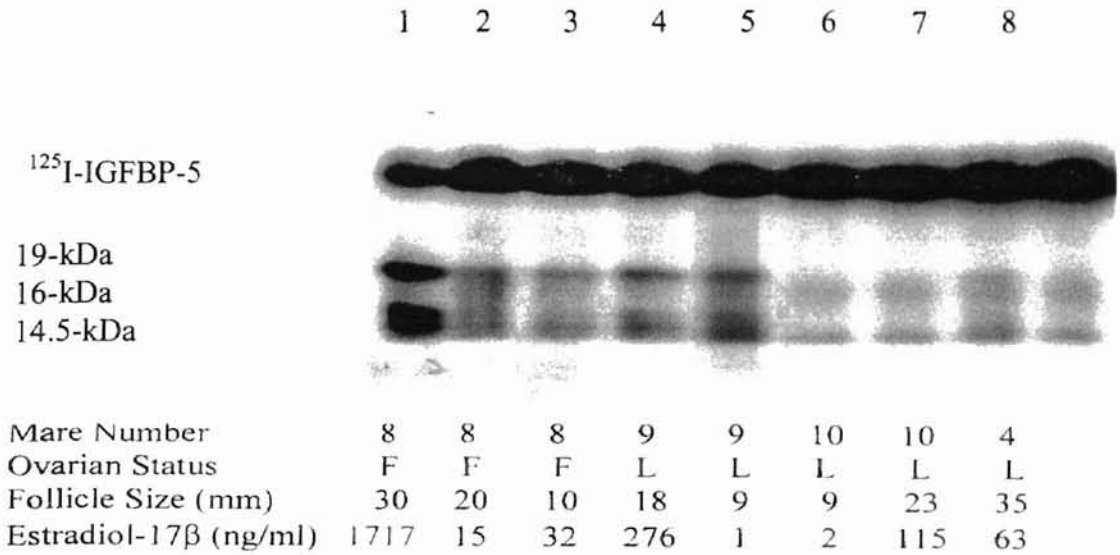


Figure 6. Representative autoradiograph measuring proteolytic activity of mare follicular fluid to recombinant human ^{125}I-labeled IGFBP-5. (F=follicular phase, L=luteal phase). Note the association of estrogenic activity in the 30 mm follicle of mare 8 with degradation of ^{125}I-IGFBP-5.

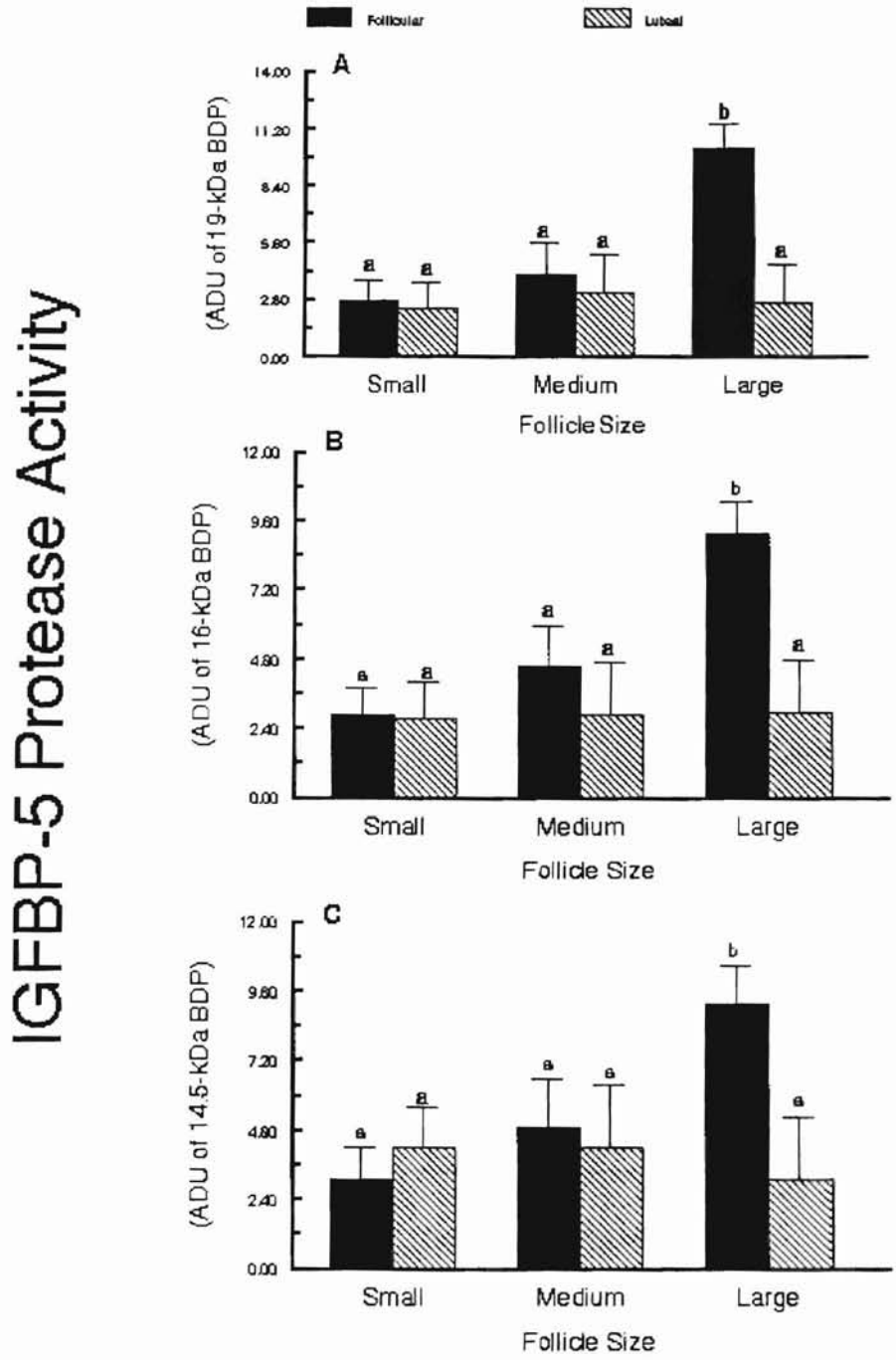


Figure 7. Mare follicular fluid protease activity to ^{125}I-IGFBP-5 as measured by the amount of breakdown products (BDP). The 19-kDa BDP (Panel A), 16-kDa BDP (Panel B), and 14.5-kDa BDP (Panel C). Follicles were classified as small (6-15 mm), medium (16-25 mm) and large (> 25 mm). Interaction of size by group ($P < 0.05$). ^{a,b} Within a panel, means without a common superscript differ ($P < 0.05$).

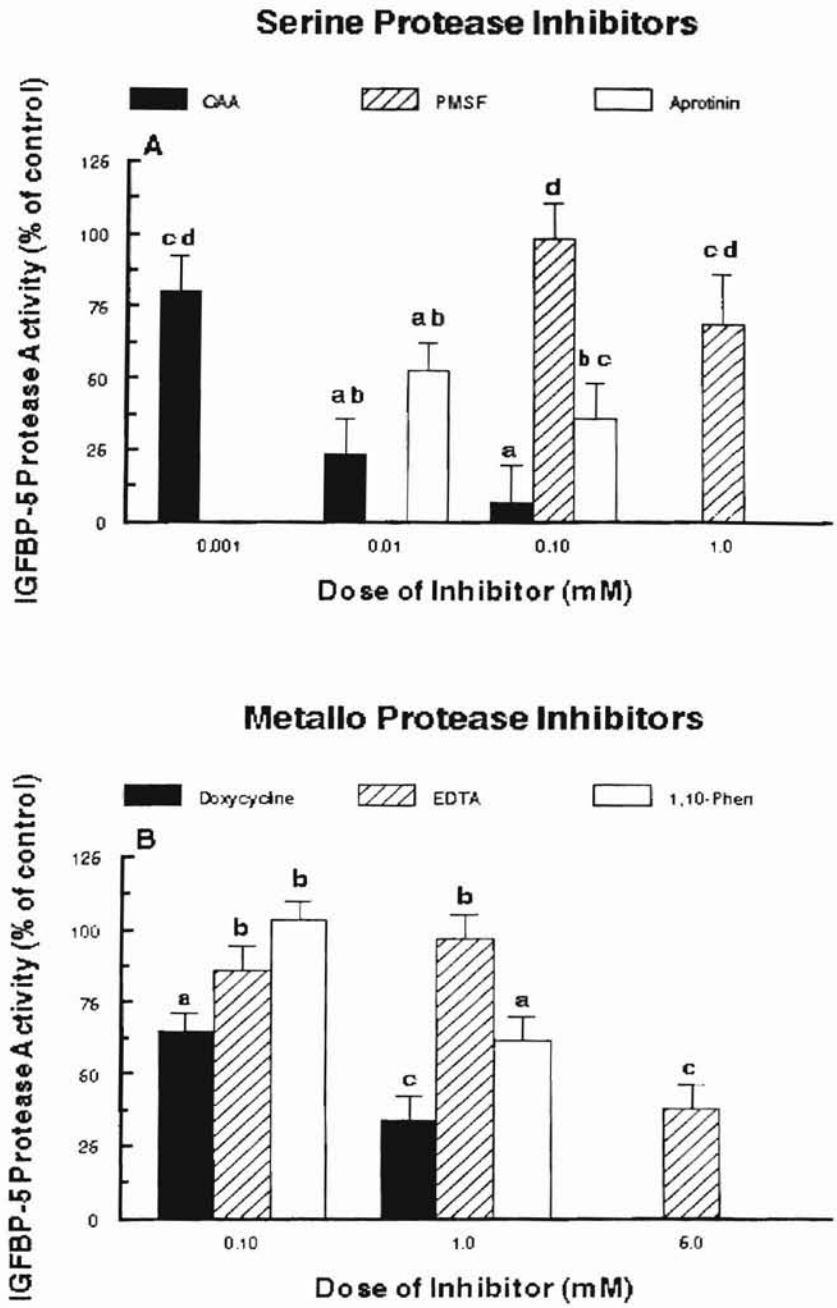


Figure 8. Inhibition of mare FF IGFBP-5 protease activity as measured by the % of control. The serine protease inhibitors CAA, PMSF and aprotinin (Panel A). The metalloprotease inhibitors doxycycline, EDTA and 1,10-Phenanthroline (Panel B). [a,b,c]Within a panel, means without a common superscript differ ($P < 0.05$).

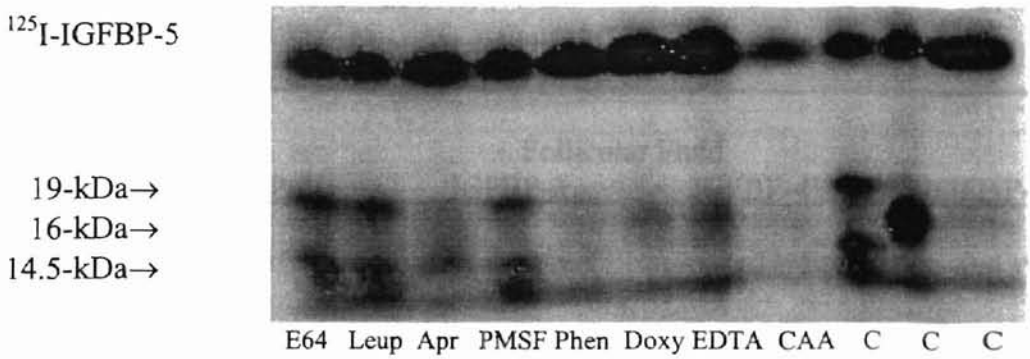


Figure 9. Representative autoradiograph measuring proteolytic activity of mare follicular fluid to recombinant human ^{125}I-labeled IGFBP-5 with protease inhibitors.

Table 1. Simple Pierson correlation coefficients among diameter, levels of estradiol-17β (E_2), androstenedione (A_4), progesterone (P_4), IGF-I, IGF-II, IGFBP-2, -3, -4, and -5 in equine follicular fluid (n=92).

	Follicular Fluid			
	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5
Diameter	-0.40**	0.18	-0.27**	-0.43**
E_2	-0.46**	0.01	-0.26*	-0.42**
A_4	-0.37**	0.02	-0.20	-0.32**
P_4	-0.18	-0.24*	-0.20	-0.33**
IGF-I	-0.73**	-0.06	-0.40**	-0.37**
IGF-II	-0.29**	-0.16	-0.009	-0.24*

*($P < 0.05$)
**($P < 0.01$)

Table 2. Simple Pierson correlation coefficients among diameter, levels of estradiol-17β, (E_2), androstenedione (A_4), progesterone (P4), IGF-I, IGF-II, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and ^{125}I-IGFBP-5 proteolytic fragments in equine follicular fluid (n=36).

	^{125}I-IGFBP-5 Fragments		
	19 kDa	17 kDa	14.5 kDa
Diameter	0.54**	0.53**	0.41*
E_2	0.68**	0.66**	0.52**
A_4	0.35*	0.34*	0.18
P_4	0.19	0.23	0.22
IGF-I	0.49**	0.52**	0.42*
IGF-II	0.45**	0.45*	0.45*
IGFBP-2	-0.53**	-0.48**	-0.39*
IGFBP-3	-0.20	-0.24	-0.33
IGFBP-4	-0.37*	-0.29	-0.31
IGFBP-5	-0.56**	-0.53**	-0.44**

*($P < 0.05$)
**($P < 0.01$)

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CHAPTER IV

SUMMARY AND CONCLUSIONS

It is thought that follicular steroidogenesis and mitogenesis are stimulated by an increase in bioavailable IGF that may occur through degradation of IGFBPs. In this study, low amounts of IGFBP-2, -4 and -5 were detected in large, estrogenic follicles when compared to small or medium follicles. As well, follicular fluid IGF-I concentrations were greater in large follicles versus small and medium follicles. IGF-II concentrations in FF were greater in follicular phase follicles than in luteal phase follicles. E_2 and A_4 concentrations in FF were greater in large than in small or medium follicles and follicular phase mares had greater E_2 in large follicles than luteal phase mares. Lack of follicular fluid proteolysis of recombinant human ^{125}I-labeled IGFBP-2 and -3 demonstrated that loss of IGFBP-2 was possibly through a decrease in gene expression in large dominant follicles. ^{125}I-labeled IGFBP-5 was cleaved by FF from large follicles collected during the follicular phase, but not the luteal phase. The IGFBP-5 proteolysis was positively correlated with follicular fluid E_2 levels. Based on results of the present study, low amounts of IGFBP-5 in follicular fluid are likely the result of enzyme degradation. This was the first evaluation on proteolysis of IGFBP-5 in mare FF. Increase in steroidogenesis of large follicles may be due to an increase in the bioavailable IGF in follicular fluid. Enzymes involved in proteolysis of IGFBP-5 are unknown and under current study.

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Master of Science

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