Effect of Follicle Size on in vitro Production of Steroids and Insulin-like Growth Factor (IGF)-I, IGF-II and the IGF Binding-Proteins (IGFBPs) by Equine Ovarian Granulosa Cells

By

TONYA RENEE DAVIDSON

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Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Traditionally, many of the major equine breed registries in the United States recognize January 1 of the year a foal was born as that foals official birth date. Thus, a foal born on March 1 and one born on August 23 would both be considered a year old on the following January 1 even though there is a considerable difference in their age. These two foals would compete on the racetrack or in the show ring with the foal born earlier in the year having an advantage. Therefore, it is desirable to breed mares so that foals will be born as soon as possible after January 1. This poses a great challenge for reproductive physiologists.

During the estrous cycle of the mare, one follicle is selected from a cohort of follicles to become dominant. After selection the dominant follicle continues to grow until ovulation, while the remaining cohort, or subordinate, follicles become atretic and regress. Limited information is available regarding the physiological mechanism of follicle selection and maturation in the mare. During preovulatory development in the mare, follicular fluid IGF-I levels increase whereas IGFBP levels decrease. In cattle, it has been hypothesized that estradiol and the gonadotropins induce changes in the amount of IGF-I and IGFBPs produced by

granulosa and thecal cells during follicular development. We hypothesize that, in the mare, follicular development may involve steroid or gonadotropin induced changes in the intrafollicular IGF system.

Therefore, the specific objectives of these experiments were to 1) determine whether stage of the estrous cycle or follicle diameter influences concentrations of steroids, as well as components of the IGF system, present in follicular fluid of mares, 2) determine whether estradiol, insulin and/or FSH affect steroid production by equine granulosa cells, and 3) determine what components of the IGF system are produced by equine granulosa cells in culture, as well as to determine whether estradiol, insulin and/or FSH affected IGF and/or IGFBP production by equine granulosa cells.

CHAPTER II

REVIEW OF LITERATURE

OVARIAN FOLLICULAR GROWTH AND DEVELOPMENT

The mare is a seasonally polyestrous, long day breeder with an ovulatory season lasting from approximately May to October in the Northern Hemisphere (Ginther, 1992). During the months of short daylength, or the anovulatory season of the mare, follicular activity is suppressed. As daylength increases, follicular activity, as indicated by an increase in the number and average diameter of follicles, increases over a period of several months (transitional period) until ovulation. During this transition period, follicles grow and regress sequentially (Ginther, 1979).

The estrous cycle of the mare lasts approximately 22 days (15 day diestrus and 7 day estrus) with ovulation occurring on the last day or two of estrus (Ginther, 1979). Ovulation in the mare is unique in that it is limited to a specific area on the ovary, the ovulation fossa, and is not triggered by a sudden release of LH as seen in other mammalian species (Whitmore et al., 1973). In contrast, LH concentrations increase progressively during estrus and peak approximately 1 day after ovulation (Whitmore et al., 1973; Stabenfeldt et al.,

1975; Alexander and Irvine, 1982). The mare is also unique in that the number of follicles involved in folliculogenesis is quite low compared to other species. For example, Driancourt and co-workers (1982b) found that the ovaries of 2 to 4 year old mares contained approximately 35,000 primordial and 100 growing follicles, whereas cow ovaries contained 120,000 primordial and 280 to 435 growing follicles (Erickson, 1966) and ewes had approximately 56,000 primordial and 150 growing follicles (Cahill et al., 1979).

Follicular Waves

Mares, like cattle, are capable of producing one or two waves of follicular activity during an estrous cycle (Evans and Irvine, 1975; Ginther, 1990). When there are adequate amounts of circulating gonadotropins, follicles are recruited from the reserve pool of small follicles (3 to 12 mm) to form major follicular waves, or waves that develop a dominant follicle (Ginther, 1992). The wave emerges as 6 mm follicles (n = 5 to 6) on approximately day 12 or mid diestrus and the follicles grow at similar rates until the largest follicle is about 23 mm. At this point, the growth rates begin to differ and one follicle, the dominant follicle, will continue to grow to approximately 35 mm in diameter, whereas the remaining follicles regress (Ginther, 1992; Ginther, 1993). Divergence of the dominant follicle is discussed in more detail in a later section of this review.

Major waves may be subdivided into primary and secondary waves. Primary waves are major waves beginning during mid-diestrus with ovulation occurring during estrus (Ginther, 1992). The day of emergence of a primary wave occurs on average 6 days after ovulation, with day of emergence being the day

before the diameters of growing follicles of a new wave exceed the diameters of the regressing follicles from a previous wave (Ginther and Bergfelt, 1993). The maximum diameter of the dominant follicle of a primary wave ranges from 36 to 53 mm (Ginther and Bergfelt, 1993). A secondary major wave may develop during the end of estrus or early diestrus (Ginther, 1992). These secondary waves occur in approximately 42% of the interovulatory intervals, however, they are more likely to occur in individual mares and some breeds (for review see Ginther, 1992).

The dominant follicle of a wave either ovulates or anovulatory follicular waves develop sequentially until conditions are right for ovulation to occur. The dominant follicle of each wave causes the regression of the subordinate cohort follicles and blocks the emergence of another wave until the dominant follicle reaches a static phase (anovulatory wave) or approaches ovulation (ovulatory wave) (Ginther, 1990). The mechanism by which the dominant follicle blocks wave emergence is still unclear, but it has been suggested that the dominant follicle produces a substance (inhibin or estradiol?) capable of suppressing circulating FSH levels thus suppressing follicular development (Miller et al., 1981; Bergfelt and Ginther, 1985). Once the growth of the dominant follicle has stopped, or ovulation approaches, another wave may emerge which contributes to regression of the dominant follicle of the previous anovulatory wave or, if ovulation has occurred, the new wave becomes the first wave of the interovulatory interval (Ginther, 1990).

Selection of the Dominant Follicle

As previously stated, approximately 12 days after ovulation a cohort (n = 5 to 6) of similar-sized (approximately 6 mm), subordinate follicles will arise (Ginther, 1979; Driancourt and Palmer, 1984; Pierson and Ginther, 1987). This cohort will grow together until the time the ovulatory follicle is selected, approximately 6 days prior to ovulation (Pierson and Ginther, 1987). Using a two-follicle model that involved ablation of all follicles ≥ 5 mm on Day 10 (Day 0 = ovulation) to allow tracking of the two largest follicles, it was determined that the future dominant follicle emerges approximately 1 day earlier than the future subordinate follicle at an approximate diameter of 6 mm (Gastal et al., 1997). These two follicles (future dominant and largest subordinate follicle) grow in parallel, with no significant difference in growth rates, until the future dominant follicle reaches a diameter of approximately 21 to 23 mm. Once the future dominant follicle reaches this critical diameter the growth rate of the smaller follicle begins to decrease. This process is called selection or deviation (Gastal et al., 1997; Gastal et al., 1999). Deviation begins and is fully established in less than 1 day as indicated by the 1-day size advantage of the largest follicle over the next largest follicle (Gastal et al., 1997), suggesting that when the largest follicle reaches a critical diameter (21 to 23 mm in the mare), it inhibits the next largest follicle before it reaches a similar critical diameter. At this time the dominant follicle begins a period of accelerated growth until ovulation (Ginther, 1979; Palmer and Driancourt, 1980; Irvine, 1981; Ginther and Pierson, 1984; Gastal et al., 1997), while the second largest follicle undergoes atresia. Other

large follicles not destined to ovulate begin to regress 6 to 7 days prior to ovulation (Driancourt et al., 1982a; Pierson and Ginther, 1987).

Studies in cattle (Ginther et al., 1996; Ginther et al., 1997) and mares (Gastal et al., 1997) indicate that when the larger follicle reaches a critical diameter (21 to 23 mm in mares) it suppresses the next largest follicle before it can reach the same diameter. The cause of this suppression is unclear, but evidence suggests that low concentrations of FSH may play a role in the deviation mechanism (for review see Ginther et al., 1996). Specifically, it has been suggested that the larger follicle suppresses FSH concentrations below the concentrations required by the smaller follicle (Gastal et al., 1999). This is supported by studies indicating that exogenous FSH administered daily at the onset of estrus (presumably prior to deviation) increases ovulation rate in mares (Squires et al., 1986; Rosas et al., 1998). Similarly, circulating FSH concentrations are low at the beginning of deviation (Bergfelt and Ginther, 1993; Gastal et al., 1997). It has not been determined if all follicles contribute to the decline in FSH concentrations before deviation in the mare, but in cattle, experimentally decreasing the number of follicles indicated that all growing follicles contribute to the FSH decline after the time of wave emergence, but prior to deviation (Gibbons et al., 1997). Involvement of estradiol in the deviation mechanism has not been ruled out. Specifically, estradiol may function by systemically inhibiting circulating FSH concentrations and by increasing LH responsiveness by the larger follicle (for review see Ginther, 1996). However, LH

is not directly involved in the initiation of deviation, but may play a role in growth and maintanence of the dominant follicle after deviation (Gastal et al., 2000).

Selection or deviation of the dominant follicle is not only a process of selecting the ovulatory follicle, but it is also a process of selection against other follicles in the cohort (Pierson and Ginther, 1990). Based on studies utilizing follicle ablation and superovulation, it has been determined that prior to dominant follicle selection, other follicles in the cohort are capable of dominance (Ginther et al., 1996; Gastal et al., 1999). Furthermore, studies in cattle indicate that other growing follicles of the wave are capable of becoming the dominant follicle if the largest follicle is destroyed between the times of emergence and shortly after the expected time of deviation (Ko et al., 1991; Gibbons et al., 1997). However, once selection has occurred the remaining follicles in the cohort are committed to atresia (Pierson and Ginther, 1990).

Initiation of Follicular Growth

The transformation of primordial to primary follicle appears to be an extremely slow process of maturation rather than one of growth (Ginther, 1992; Gougeon, 1996). Evidence in primates suggest that recruitment of primordial follicles from the reserve pool of resting follicles begins by conversion of flattened, squamous-type granulosa cells of primordial follicles into cuboidal, epithelial type cells, thus increasing follicle diameter (Gougeon and Chainy, 1987; Gougeon, 1996). Moreover, the number and activity of granulosa cells in

relation to diameter of the oocyte at initiation appears to be highly species specific (McNatty et al., 1999).

The signal for initiation of follicular growth remains to be elucidated, however several hypotheses exist. In the mouse, inhibition of binding of the tyrosine kinase receptor c-kit with its ligand, stem cell factor, prevents follicular initiation without blocking the formation of primordial follicles (Huang et al., 1993; Yoshida et al., 1997). Moreover, administration of a c-kit antibody to mice stopped granulosa cell proliferation (Yoshida et al., 1997), and initiation failed to occur in mice mutant for the kit ligand gene despite the presence of primordial follicles (Kuroda et al., 1988; Huang et al., 1993). Other studies in the mouse suggest that initiation of follicle growth is dependent on collagen and laminin, but not on activin-A (Oktay et al., 2000). Research in sheep suggests that two major genes are involved in growth initiation. Specifically, ewes homozyous for the Inverdale prolificacy gene exhibit abnormal follicular and oocyte growth suggesting that this gene influences initiation of follicular growth, particularly by inhibiting growth at the primordial/primary stage (Bodensteiner et al., 2000). Furthermore, ewes homozygous for the Belclare gene are sterile due to an alteration in granulosa cell proliferation (Reynaud et al., 1999). Recent studies in the primate suggest that androgen treatment increases the number of primary follicles in the primate ovary perhaps by stimulating an increase in both IGF-I and IGF-I receptors thus triggering oocyte development and initiation of follicle growth (Vendola et al., 1999). Further studies are needed to determine if any of these mechanisms may be involved in initiation of follicular growth in the mare.

Follicular Growth and Atresia

Follicular growth is initiated from a reserve of small follicles consisting of resting follicles (follicles with an oocyte area smaller than 180 μm^2), transitional follicles (follicles with an oocyte nuclear area between 180 and 250 μm^2) that form a transitional population between the resting and growing follicles (follicles with an oocyte nuclear area exceeding 250 μm^2 and a follicular area larger than 2000 μm^2 ; Driancourt et al., 1982b). Follicular growth occurs by both proliferation of granulosa cells and by an increase in the size of the oocyte (Gougeon, 1996). As the primary follicle progresses toward a secondary follicle the stromal cells near the basal lamina align parallel to one another, and the surrounding connective tissue stratifies and differentiates into the theca externa (similar to the undifferentiated cell type) and the theca interna (characterized by a change from fibroblast-like precursor cells to epitheliod cells) (Gougeon, 1996). In equine follicles a well-defined theca layer can not be detected until follicles reach 0.13 mm in diameter (Driancourt et al., 1982b). Once epitheliod cells appear in the theca interna, the follicle is no longer classified as a secondary follicle, but rather a preantral follicle (Gougeon, 1996). When the follicle reaches a mean of approximately 0.3 mm in diameter (Driancourt et al., 1982b), formation of the antral cavity begins by the development of small, fluid filled cavities that aggregate to form the antrum (for review see Hillier, 1994). Antral formation is dependent on stimulation by FSH, which acts via receptors on the granulosa cell to stimulate cell division and synthesis of glycosaminoglycans, essential components of follicular fluid (for review see Hillier, 1994). The number of these

small antral follicles (2 to 5 mm follicles in the mare) is not constant throughout the estrous cycle, but begins to increase around the time of ovulation (Pierson and Ginther, 1987). Once the antrum forms the granulosa cells surrounding the oocyte become known as the cumulus oophorus and the granulosa cells near the basement membrane differentiate from cuboidal to columnar cells (Gougeon, 1996). Fluid accumulation within the antral cavity, and proliferation of granulosa and theca interstitial cells continue until the follicle reaches a size suitable for selection as the dominant follicle (25 to 30 mm in the mare; Pierson and Ginther, 1987; Gougeon, 1996).

The pituitary gonadotropins, LH and FSH, are important regulators of folliculogenesis. Each follicle in the cohort of developing follicles must be stimulated by FSH to begin development, otherwise it will undergo atresia (for review see Hillier, 1994). Once the threshold for FSH stimulation is surpassed, granulosa cell genes encoding aromatase, LH receptor and α-inhibin are increasingly expressed, mediated by second-messenger cyclic AMP (for review see Hillier, 1994). The follicle with the lowest FSH threshold will become the first follicle to produce estradiol (Hillier, 1981). Increasing estradiol will suppress FSH secretion in a negative feedback mechanism with the hypothalamic-pituitary axis. Circulating FSH levels will decrease to levels insufficient for development of other follicles in the cohort (Hillier, 1981). Once the dominant follicle has been selected, it is dependent on LH to maintain its status as dominant follicle. This is accomplished through a paracrine signaling system that remains to be elucidated; however inhibin has been implicated as an important factor (Hillier,

1991). Toward the end of the follicular phase, plasma FSH levels begin to decline and the LH surge suppresses granulosa cell proliferation and intitiates atresia of subordinate follicles and luteinization of preovulatory follicles (for review see Hillier, 1994).

Driancourt et al. (1982b) suggested that in the mare approximately 75% of follicles are undergoing atresia at any given time; however atresia was rarely seen before follicles reached a medium antral size. The mechanism that determines if a follicle is to become atretic remains to be determined. Approximately six days prior to ovulation, coincident with the time of selection, all follicles, with the exception of the dominant follicle, begin to regress (Ginther and Pierson, 1984; Pierson and Ginther, 1987). It has been suggested that the dominant follicle of each wave causes atresia of the cohort of subordinate follicles and blocks emergence of the next follicular wave until ovulation occurs (Ginther, 1990). Emergence of the next follicular wave induces regression of the dominant follicle of an anovulatory wave (Ginther, 1990).

Follicular Steroidogenesis

Folliculogenesis is mainly under endocrine control of the pituitary gonadotropins, LH and FSH (for reviews see Combarnous 1992; Adashi, 1994b; Hillier, 1994). By binding to specific receptors on ovarian cells, the gonadotropins stimulate synthesis and secretion of steroids that will act as endocrine and paracrine regulators of gonadotropin levels, ovarian activity and follicular development (for reviews see Gougeon, 1996). Steroidogenesis is dependent on

the availability of cholesterol for conversion to pregnenolone, progesterone, androgens and estrogens (for review see Conley and Baird, 1997). Steroidogenic acute-regulatory protein (StAR) acts to transfer cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane to allow the enzymatic conversion of cholesterol to pregnenalone via cholesterol side-chain clevage ($P450_{scc}$). Pregnenalone can then be metabolized by either the $\Delta 4$ or $\Delta 5$ pathway; the $\Delta 4$ pathway is preferential in equine follicles (Younglai and Short, 1970). The $\Delta 4$ pathway involves conversion of pregnenalone to progesterone via 3β-hydroxysteroid dehydrogenase (3βHSD). Progesterone is then converted to androstenedione via 17α-hydroxylase ($P450_{17\alpha}$) in the endoplasmic reticulum. 17β-hydroxysteroid dehydrogenase (17βHSD) then catalyses the conversion of androstenedione to testosterone, which can then be converted to estrogen via aromatase.

Steroidogenesis in the mare involves both granulosa and theca interna cells as in the classic "two-cell theory" of follicular steroidogenesis (Senger, 1997). Briefly, when stimulated by LH the theca interna produces androgens that are converted to estrogens in the granulosa cells when stimulated by FSH via the aromatizing enzyme, aromatase. Granulosa cells in the mare follicle are abundant in 3β-hydroxysteroid dehydrogenase (required for the conversion of pregnenolone to progesterone) and aromatase (Jarrell and Robaire, 1982). The presence of aromatase indicates that isolated granulosa cells are capable of producing estradiol. In contrast, theca interna (Sirois et al., 1991; Rodger et al., 1995), but not granulosa (Belin et al., 2000) cells appear to be rich in enzymes

such as cholesterol side-chain cleavage ($P450_{scc}$), 17α-hydroxylase ($P450_{17\alpha}$), and 3β-hydroxysteroid dehydrogenase (3βHSD), indicating that the theca interna is the predominant site for androstenedione synthesis in equine preovulatory follicles. The presence of $P450_{17\alpha}$ in the theca interna (Sirois et al., 1991; Rodger et al., 1995) of equine follicles is similar to observations in cattle (Arlotto et al., 1996), pigs (Conley and Baird, 1997; Tian et al., 1995) and sheep (Huet et al., 1997).

In many species, large dominant follicles are capable of producing large amounts of estradiol-17β, whereas atretic follicles produce decreasing amounts of estradiol-17β (cows – Ireland and Roche, 1982; pigs – Guthrie et al., 1993; humans – McNatty and Baird, 1978; Hillier et al., 1981; sheep – Carson et al., 1981). Similarly in the mare, estradiol-17β and progesterone levels increase during follicular growth, with maturation characterized by a decrease in estradiol-17β and a further increase in progesterone levels (Gerard et al., 1998; Gerard and Monget, 1998; Belin et al., 2000). Increased steroidogenic activity may be the result of increased levels of steroidogenic enzymes such as $P450_{scc}$, 3βHSD and aromatase (Belin et al., 2000). Moreover, subordinate follicles are characterized by a decrease in $P450_{scc}$, 3βHSD and aromatase, as well as decreased levels of estradiol-17β and progesterone (Almadhidi et al., 1995; Gerard and Monget, 1998; Belin et al., 2000). Thus, it appears that changes in intrafollicular steroids during folliculogenesis are determined by expression of the various steroidogenic enzymes (Belin et al., 2000).

OVARIAN INSULIN-LIKE GROWTH FACTORS, THEIR BINDING PROTEINS, AND RECEPTORS

Follicular development is controlled by numerous endocrine and paracrine factors. Of the paracrine factors involved, the insulin-like growth factor system has been implicated in modulation of gonadotropin action on proliferation and differentiation of granulosa and thecal cells (Monget et al., 1996). The IGF system is composed of the following

- two ligands, IGF-I and –II (Humbel, 1990)
- two receptors, a type I receptor responsible for mediation of IGF-I and –II action (Roth and Kiess, 1994), and a type II receptor or IGF-II/mannose-6-phosphaste (IGF-II/M6P) receptor that appears to be involved in IGF-II degradation (Monget and Bondy, 2000).
- at least six high affinity binding proteins (IGFBP-1 through –6) (Rajaram et al., 1997).

Insulin-like Growth Factor (IGF)-I and –II

IGF-I and –II were first isolated from human serum by Rinderknecht and Humbel in 1978 (Rinderknecht and Humbel, 1978a,b). Both are single chain polypeptides with three intrachain disulfide bonds. IGF-I consists of 70 amino acids with a molecular mass of 7.65 kDa, while IGF-II consists of 67 amino acids with a molecular mass of 7.47 kDa. IGF-I and –II are over 60% homologous with each other but less homologous with insulin and its precursor, proinsulin. Furthermore, the amino acid sequence of both IGF-I and –II are highly

homologous (i.e., >90%) across species (Spicer and Echternkamp, 1995). IGF-I, -II and proinsulin are all composed of A and B insulin regions joined by two disulfide bonds and a connecting C-peptide. The IGF-I gene is expressed as a prepro IGF-I that requires post-translational modification to clip off both the amino terminal signal peptide and the carboxy terminus E peptide to produce the 70 amino acid form of IGF-1 common in biological tissues and fluids (for reviews see Baxter, 1988b; Rosenfeld et al., 1990). The human IGF-I gene is approximately 100 kilobases found on the long arm of chromosome 12 (Brissenden et al., 1984; Tricoli et al., 1984) whereas the human IGF-II gene is on the short arm of chromosome 11, near the insulin gene, and consists of approximately 30 kilobases (Brissenden et al., 1984; Tricoli et al., 1984; Bell et al., 1985).

Both IGF-I and IGF-II are synthesized and secreted in a wide variety of organs and tissues including the ovary. The IGFs have been detected in follicular fluid of pigs (Spicer et al., 1992; Echternkamp et al., 1994b), cattle (Spicer et al., 1988; Spicer and Enright, 1991; Kirby et al., 1993), mares (Spicer et al., 1991), women (Jesionowska et al., 1990) and sheep (Spicer et al., 1995). Moreover, IGF-I and –II mRNA have been detected in ovarian tissue of cattle (Einspanier et al., 1990; Spicer et al., 1993; Yuan et al., 1998), human (Voutilainen and Miller, 1987; Barreca et al., 1993; El-Roeiy et al., 1993; El-Roeiy et al., 1994), sheep (Spicer et al., 1995), pigs (Samaras et al., 1994) and rats (Murphy et al., 1987; Hernandez et al., 1989; Oliver et al., 1989). Specifically, in rats, IGF-I mRNA is found almost exclusively within granulosa cells, whereas IGF-II mRNA is found in thecal cells (Hernandez et al., 1989; Oliver et al., 1989). IGF-I mRNA has been

found in granulosa cells of pigs (Hatey et al., 1992) and in both granulosa and thecal cells of cattle (Spicer et al., 1993; Spicer and Echternkamp, 1995). Moreover, granulosa cells of cattle (Spicer et al., 1993; Spicer and Chamberlain, 2000) and pigs (Hsu and Hammond, 1987a,b) secrete IGF-I.

In addition to being species specific, the IGF gene is expressed in a cell-specific pattern that is dependent on the developmental stage of the follicle (Lucy, 2000). For example, IGF-I gene expression is localized within the granulosa cells of healthy, developing follicles in mice (Adashi et al., 1997), with maximum levels found in granulosa cells of large preantral follicles and in the cumulus of small antral follicles (Wandji et al., 1998). Moreover, small antral follicles (<4 mm) of cattle (Echternkamp et al., 1990; Spicer and Enright, 1991) and pigs (Hammond et al., 1988; Spicer et al., 1992; Echternkamp et al., 1994b) had lower concentrations of IGF-I than did large (>5 mm) antral follicles, and IGF-I and –II mRNA were greater in granulosa cells of dominant bovine follicles than in subordinate follicles during the first follicular wave (Yuan et al., 1998). Studies in IGF-I knock-out mice suggest that an increase in IGF-I gene expression in large preantral follicles stimulates an increase in FSH receptors and an increase in type I IGF receptors which ultimately amplify FSH action, formation of the antrum, and expression of aromatase and LH receptors in fully differentiated follicles (Zhou et al., 1997). This positive feedback loop has also been seen in sheep (Perks et al., 1995) and cattle (Wandji et al., 1992). In contrast, IGF-I mRNA did not change as follicles grew from 2 to 8 mm (preovulatory size) in weaned sows; however, IGF-II mRNA increased as follicles

developed to 6 mm in diameter, but then decreased as follicles developed to 8 mm in diameter (Liu et al., 1999). These results are similar to those found in cyclic sows where IGF-I mRNA levels did not change from small to large antral follicles, while IGF-II mRNA levels increased as follicles grew (Yuan et al., 1996).

In vitro, IGF-I stimulates both granulosa and thecal cell proliferation and differentiation of several species (for review see Giudice, 1992; Monget and Monniaux, 1995; Spicer and Echternkamp, 1995). Specifically, IGF-I acts synergistically with gonadotropins to enhance progesterone and(or) estradiol production by granulosa cells of cattle (Spicer et al., 1993; Armstrong et al., 1996; Spicer and Chamberlain, 1999), humans (Erickson et al., 1989; Iwashita et al., 1996) and rats (Adashi et al., 1984). In the absence of FSH, IGF-I is a weak stimulator of steroidogenesis but potent stimulator of granulosa cell proliferation (Spicer et al., 1993; Armstrong et al., 1996; Spicer and Chamberlain, 1999). It has been hypothesized that the IGF-I induced FSH amplification may distinguish follicles destined to ovulate from those destined for atresia (Adashi, 1994a). In recent in vitro studies, IGF-I in combination with FSH decreased the occurrence of spontaneous apoptosis in porcine granulosa cells (Guthrie et al., 1998). IGFs may be required for entry of follicles into the pool of growing follicles and for their gonadotropin-independent development, whereas, entry of follicles into the gonadotropin-dependent stage of follicular development may require an increase in FSH receptors and/or type I IGF receptors in cattle (Wandji et al., 1992), and IGF-II and/or type-I IGF receptors in sheep (Perks et al., 1995). Studies utilizing IGF-I null mice supply the first real evidence of the involvement of the IGF

system in folliculogenesis and fertility. These studies indicate that IGF-I is permissive but not crucial for the recruitment of primordial follicles, the growth of preantral follicles, and cell proliferation, at least up to the antral stage of follicular development (Baker et al., 1996). Similar results have been found in cattle (Gong et al., 1991; Chase et al., 1998).

Insulin-like Growth Factor Binding Proteins

IGFBP-1 through –6 share ~35% sequence identity with one another (Rajaram et al., 1997). They each contain a cysteine rich region at both the amino and carboxy terminal regions of the protein as well as 18 conserved cysteines (with the exception of human IGFBP-2 which has 20) arranged in approximately the same spatial configuration (Rosenfeld et al., 1990). Conservation of this spatial configuration is indicative of a well-conserved secondary structure of the IGFBPs (Rosenfeld et al., 1990). There is also a conserved Arg-Gly-Asp sequence near the carboxy terminal of all IGFBPs with the exception of human IGFBP-3 (Rosenfeld et al., 1990). However, there are significant differences in the "spacer" regions between the conserved cysteines as well as in the signal peptide sequences and in glycosylation of the IGFBPs (Wood et al., 1988; Zapf et al., 1988; Lamson et al., 1989b). The IGFBP genes are members of a large gene family (Rosenfeld et al., 1990) with human IGFBP-1 and –3 genes located on chromosome 7 (Ehrenborg et al., 1992), IGFBP-2 and –5 on chromosome 2 (Allander et al., 1993; Allander et al., 1994), and IGFBP-4

and -6 on chromosomes 17 and 12, respectively (Ehrenborg et al., 1992; Allander et al., 1994).

IGFBP-1

IGFBP-1 is a 25 to 34 kDa (for review see Spicer and Echternkamp, 1995) nonglycosylated, acid stabile protein (Ooi and Herington, 1988) first isolated from mid term human amniotic fluid (Drop et al., 1979). It binds IGF-I and –II with equal affinity and acts to inhibit and/or potentiate IGF action (Rajaram et al., 1997). The amino acid sequence of IGFBP-1 consists of 12 N-terminal and 6-C terminal cysteine residues (Lee et al., 1997) and is 68 to 72% homologous among cattle, mice, rats and humans (Chamberlain, 1999). The amino acid sequence of equine IGFBP-1has not been determined.

IGFBP-1 is the primary binding protein in amniotic fluid (Giudice et al., 1991) and is also present in human plasma (Rabinovici et al., 1997), serum (Hartshorne et al., 1990) and follicular fluid (Harstshorne et al., 1990), as well as milk, urine, synovial fluid, interstitial fluid and seminal fluid of humans (for review see Rajaram et al., 1997). IGFBP-1 mRNA has been localized in the placenta (Brewer et al., 1988; Lamson et al., 1989b) and some breast cancer cell lines (Yee et al., 1989) of women, and is the most prominently expressed IGFBP in decidualized, and to a lesser extent secretory, endometrial tissue (Brewer et al., 1988).

Although IGFBP-1 is present in follicular fluid, only a few studies have been conducted to determine if the ovary or follicle produce it. IGFBP-1 is

present in the ovary of women, but to date has not been identified in ovarian tissue of other species. IGFBP-1 has been found in human luteinized cells of hyperstimulated preovulatory follicles and cells of the corpus luteum (Seppala et al., 1984). In vitro, IGFBP-1 has been found in cultured human stromal and thecal cells (Mason et al., 1991), luteinized granulosa cells (Suikkari et al., 1989; Giudice et al., 1991), and in human granulosa cell-conditioned medium (Suikkari et al., 1989). Human granulosa-luteal cells contain and express mRNA for IGFBP-1 (Seppala et al., 1984; Giudice et al., 1991) whereas thecal cells contain IGFBP-1 mRNA, but not the protein (Jalkanen et al., 1989). IGFBP-1 is not produced by porcine granulosa cells, nor has IGFBP-1 mRNA detected by Northern blot analysis (Grimes et al., 1994a). Because human granulosa cells contain and express IGFBP-1 mRNA it is likely that these cells contribute to the amount of IGFBP-1 present in follicular fluid, at least in women.

IGFBP-2

IGFBP-2 is a 29 to 40 kDa (for review see Spicer and Echternkamp, 1995) nonglycosylated, acid stabile protein (Ooi and Herington, 1988) first isolated in from rat liver (BRL)-3A cell line (Mottola et al., 1986). It binds IGF-II with greater affinity than IGF-I and acts as an inhibitor of IGF action (Rajaram et al., 1997). The amino acid sequence of IGFBP-2 is 64 to 92% homologous among cattle, mice, rats, pigs, humans and sheep (Chamberlain, 1999). The amino acid sequence of equine IGFBP-2 has not been determined.

IGFBP-2 is the primary binding protein in the central nervous system, cerebrospinal fluid (Binoux et al., 1982) and in neonatal rat serum (Giudice et al.,

1991). It has also been found in adult rat serum (Shimasaki et al., 1991b), human milk, follicular fluid and seminal plasma (Binoux et al., 1991a,b), rat brain and cultured neuron and glial cells (Lamson et al., 1989a), as well as bovine endometrium and, to a lesser extent, myometrium (Kirby et al., 1996).

Because IGFBP-2 is present in follicular fluid, other studies have been conducted to determine if the ovary or follicle produce it. IGFBP-2 mRNA is the primary binding protein mRNA present in the human ovary and is localized within both granulosa and thecal cells (Voutilainen et al., 1996). Moreover, IGFBP-2 has been detected in human granulosa cell conditioned media (Cwyfan Hughes et al., 1997). In cattle, IGFBP-2 mRNA has been localized to granulosa cells (Yuan et al., 1998) and cells of the corpus luteum (Kirby et al., 1996), whereas IGFBP-2 protein has been detected in both granulosa and thecal cells (Funston et al., 1996) as well as bovine follicular fluid. In other species, IGFBP-2 mRNA has been localized within granulosa and thecal cells and corpora lutea of pigs (Samaras et al., 1992) and sheep (Perks and Wathes, 1996) and in theca intersitial and secondary interstitial cells of the rat (Nakatani et al., 1991). Collectively, these findings suggest that IGFBP-2 produced by follicular cells contribute significantly to follicular fluid concentrations of IGFBP-2.

IGFBP-3

IGFBP-3 is a 28 kDa to 53 kDa (Wood et al., 1988; Spicer and Echternkamp, 1995) N-glycosylated protein, and is the major IGFBP present in human circulation (Baxter et al., 1986) and follicular fluid (Cataldo and Giudice,

1992a). In circulation, IGFBP-3 forms a 150- to 200-kDa complex consisting of IGFBP-3, IGF- I or -II and an acid-labile subunit (Baxter, 1988a; Baxter, 1990). IGFBP-3 binds IGF-I and IGF-II with equal affinity and acts as an inhibitor and/or potentiator of IGF action (Rajaram et al., 1997). The amino acid sequence of IGFBP-3 is 78 to 89% homologous among cattle, mice, rats, humans and pigs (Chamberlain, 1999). The amino acid sequence of equine IGFBP-3 has not been determined.

In mammals, IGFBP-3 is present in serum, follicular fluid, milk, urine, cerebrospinal fluid, amniotic fluid, synovial fluid, interstitial fluid and seminal fluid (for review see Rajaram et al., 1997). The main site of IGFBP-3 gene expression is in the liver (Wood et al., 1988) but has also been detected in bovine (Kirby et al., 1996) and human (Lamson et al., 1989b) endometrium.

Because IGFBP-3 is present in follicular fluid, numerous studies have been conducted to determine if the ovary or follicle produce it. In humans, IGFBP-3 is present in granulosa (Cataldo et al., 1993) and thecal (Hughes et al., 1997) cells, thecal conditioned media, and atretic follicles (Cwyfan-Hughes et al., 1997). IGFBP-3 mRNA has been localized in cultured human granulosa cells (Giudice et al., 1991), as well as granulosa and thecal cells of dominant human follicles (El-Roeiy et al., 1994). However, in small antral follicles of women IGFBP-3 mRNA has been detected only in thecal cells (El-Roeiy et al., 1994). In rats, IGFBP-3 mRNA has been detected in regressing corpora lutea (Erickson et al., 1993), but not in granulosa or thecal cells (Nakatani et al., 1991). Localization of IGFBP-3 mRNA in the porcine ovarian compartment is similar to that of the rat

except that low levels of IGFBP-3 mRNA have been detected in thecal cells (Samaras et al., 1992) and IGFBP-3 protein is produced by porcine granulosa cells (Grimes et al., 1994a). In cattle, IGFBP-3 mRNA is localized in granulosa but not thecal cells of small antral follicles (Yuan et al., 1998). Because follicular fluid levels of IGFBP-3 remain constant throughout the human menstrual cycle (Cataldo and Giudice, 1992b; Huang et al., 1994) and the estrous cycle of pigs (Grimes et al., 1994b), cattle (Echternkamp et al., 1994a; de la Sota et al., 1996; Funston et al., 1996; Stewart et al., 1996), sheep (Monget et al., 1993; Spicer et al., 1995) and horses (Gerard and Monget, 1998), it is unlikely that intrafollicular production of IGFBP-3 contributes significantly to the amount of IGFBP-3 present in follicular fluid.

IGFBP-4

IGFBP-4 is a 25 to 30 kDa (for review see Spicer and Echternkamp, 1995) nongycosylated protein first isolated from human osteosarcoma TE-89 cell conditioned medium (Mohan et al., 1989) and from adult rat serum (Shimonaka et al., 1989). IGFBP-4 binds IGF-I and IGF-II with equal affinity and acts as an inhibitor of IGF action (Rajaram et al., 1997). The amino acid sequence of IGFBP-4 is 88 to 98% homologous among cattle, pigs, mice, rats, humans and sheep (Chamberlain, 1999). The amino acid sequence of equine IGFBP-4 has not been determined.

IGFBP-4 has been detected in serum, follicular fluid, seminal fluid, interstitial fluid and synovial fluid (for review see Rajaram et al., 1997) as well as

conditioned media of normal human osteoblast-like cells (Mohan et al., 1989), fibroblasts (Camacho-Hubner et al., 1992), endometrial stromal cells (Irwin et al., 1995) and neuroblastoma cells (Fielder et al., 1990; Cheung et al., 1991).

Because IGFBP-4 is present in follicular fluid, other studies have been conducted to determine if the ovary or follicle produce it. Within the human ovary, IGFBP-4 protein is primarily localized in granulosa cells of atretic follicles (Cataldo et al., 1993; Liu et al., 1993; Voutilainen et al., 1996), but has also been detected in granulosa and thecal cells of antral follicles of polycystic ovarian syndrome women (El-Roeiy et al., 1994). IGFBP-4 mRNA has been localized in granulosa and thecal cells of large (14 mm) human follicles, but is more abundant in granulosa cells of small (11 mm) follicles (Voutilainen et al., 1996). In addition, IGFBP-4 mRNA has been detected in human luteal cells and in oocytes from preantral to antral stages of development (Peng et al., 1996). In rats, IGFBP-4 protein is found in granulosa cells of atretic follicles and IGFBP-4 mRNA was weakly expressed in dominant follicles (Liu et al., 1993). In pigs, IGFBP-4 protein is present in atretic follicles as well as small healthy follicles (Besnard et al., 1997). Porcine IGFBP-4 mRNA has been localized to thecal cells of medium size growing follicles, luteinizing granulosa cells (Gadsby et al., 1996; Zhou et al., 1996) and luteal cells (Zhou et al., 1996). In sheep, IGFBP-4 mRNA has been localized in both granulosa and thecal cells of dominant follicles (Besnard et al., 1996a). IGFBP-4 mRNA has also been detected in granulosa and thecal cells of atretic bovine follicles (Armstrong et al., 1998). Therefore, it is likely that intrafollicular production of IGFBP-4 contributes significantly to the

amount of IGFBP-4 present in follicular fluid; however, species differences exist. Moreover, IGFBP-4 is likely a key factor in follicular atresia.

IGFBP-5

IGFBP-5 is a 29 to 31 kDa (for review see Spicer and Echternkamp, 1995) O-linked glycosylated (Conover and Kiefer, 1993) protein first isolated from adult rat serum, human bone extract and U-2OS human osteosarcoma cell line conditioned medium (Shimasaki et al., 1991a; Bautista et al., 1991). IGFBP-5 binds IGF-II with greater affinity than IGF-I and acts as a potentiator of IGF action (Rajaram et al., 1997). The amino acid sequence of IGFBP-5 is 97 to 99% homologous among cattle, mice, rats, pigs and humans (Chamberlain, 1999). The amino acid sequence of equine IGFBP-5 has not been determined.

IGFBP-5 has since been detected in serum, follicular fluid and cerebrospinal fluid (for review see Rajaram et al., 1997) and mRNA has been localized in numerous cells and tissues including rat liver, brain, lung, adrenal, spleen, heart, kidney, intestine, stomach and testis homogenates (Erickson et al., 1992), and human fibroblasts (Camacho-Hubner et al., 1992) and osteoblast (Conover and Keifer, 1993; Canalis and Gabbitas, 1995) cells.

Because IGFBP-5 is present in follicular fluid, other studies have been conducted to determine if the ovary or follicle produce it. IGFBP-5 mRNA has been detected in human granulosa and thecal cells (Voutilainen et al., 1996). In pigs, IGFBP-5 protein is secreted by granulosa cells (Grimes et al., 1994a), however, no IGFBP-5 mRNA has been detected in porcine follicles (Zhou et al.,

1996), suggesting that in pigs IGFBP-5 is not an important IGFBP in terms of follicular selection or function. In sheep, IGFBP-5 mRNA has been localized in granulosa cells of atretic follicles and thecal cells of healthy follicles (Besnard et al., 1996a). Similarly in the rat, IGFBP-5 mRNA is present in granulosa cells of atretic follicles, secondary interstital cells and luteal cells (Erickson et al., 1992). Moreover, IGFBP-5 mRNA has been detected in cultured rat granulosa cells (Liu et al., 1993). Therefore, It is likely that intrafollicular production of IGFBP-5 contributes significantly to the amount of IGFBP-5 present in follicular fluid; however, species differences exist.

IGFBP-6

IGFBP-6 is a 21 to 32 kDa (for review see Spicer and Echternkamp, 1995) O-glycosylated (Shimasaki et al., 1991a) protein first purified from transformed human fibroblast cell cultures (Martin et al., 1990) and from human cerebrospinal fluid (Bach et al., 1992; Bach et al., 1994). IGFBP-6 binds IGF-II with greater affinity than IGF-I and acts as an inhibitor of IGF action (Rajaram et al., 1997). The amino acid sequence of IGFBP-6 is 67 to 83% homologous among cattle, rats, mice, and humans (Chamberlain, 1999). The amino acid sequence of equine IGFBP-6 has not been determined.

IGFBP-6 is primarily found in humans and rats. It has been localized in many body tissues and fluids including serum (Zapf et al., 1990), retinal pigment epithelial cells (Feldman and Randolph, 1994), prostate cancer cells (Srinivasan

et al., 1996), osteoblasts (Gabbitas and Canalis, 1997), amniotic fluid (Rajaram et al., 1997) and bovine mammary epithelial cells (Cohick and Turner, 1998)

IGFBP-6 mRNA has been localized in human granulosa, thecal and stromal cells (Voutilainen et al., 1996) and rat granulosa and thecal interstitial cells (Rohan et al., 1993). Although IGFBP-6 is present in porcine follicular fluid (Shimasaki et al., 1990), IGFBP-6 is not produced by porcine granulosa cells (Grimes et al., 1994a). Moreover, IGFBP-6 mRNA has not been detected in porcine granulosa cells by Northern blot analysis (Grimes et al., 1994a). This suggests that in swine, intrafollicular levels of IGFBP-6 are most likely produced by other types of follicular cells. To date, IGFBP-6 does not appear to be a major contributor to intraovarian IGFBP content in most species.

New IGFBPs

Recently, several new gene products have been suggested to be part of the IGFBP family. The mac25 gene, encoding a 277 amino acid preprotein, has been cloned and sequenced from leptomeningial and mammary epithelial cells (Swisshelm et al., 1995). The mature protein has not yet been identified, but the deduced amino acid sequence of the mac25 propeptide shows 40 to 45% similarity and 20 to 25% identity to IGFBPs (Oh et al., 1996). The mac25 propeptide contains the common IGFBP motif (GCGGCCXXC) at the amino terminus, in an area containing a cluster of 12 conserved cysteines, 11 of which are found in mac25 (Oh et al., 1996). Thus, mac25 meets structural criteria to be considered another member of the IGFBP family. Moreover, mac25 specifically

binds IGF-I and –II and insulin, but with rather low affinity (Oh et al., 1996). Therefore, mac25 has been named IGFBP-7. Similarly, a family of closely related gene encoding connective tissue growth factor (CTGF; Bradham et al., 1991), the *nov* oncogene (Martinerie et al., 1992) and *cyr61* (O'Brien et al., 1990) have been identified and suggested to be members of the IGFBP family. These proteins have 30 to 38% sequence identity with IGFBP-1 through –6. Furthermore, these proteins contain the characteristic IGFBP motif (GCGCCXXC) in their amino terminus and 17 of the 18 cysteines are conserved (Bork, 1993). CTGF has also been found to bind IGF-I and –II, although with relatively low affinity (Kim et al., 1997). CTGF, *nov,* and *cyr61* have been named IGFBP-8, -9 and –10, respectively (Kim et al., 1997). However, Baxter and coworkers (1998) have suggested that these proteins not be named IGFBPs, but insulin-like growth factor-related proteins (IGFBP-rP) until their molecular, biochemical and physiological relationships can be more closely related to the IGFBPs.

IGFBP actions within the ovary

In general, each IGFBP has a specific function that is determined by structural differences of the binding protein (for example, glycosylation, number of cysteines, RGD sequence), binding affinity and tissue-specific expression (Rajaram et al., 1997). In particular, each IGFBP may either stimulate or inhibit IGF action depending on posttranslational modifications such as phosphorylation (Koistinen et al., 1993), proteolysis and cell surface association (Hossenlopp et

al., 1990; Conover, 1992; Frost et al., 1993; Lalou and Binoux, 1993; Liu et al., 1993). These factors act by altering the binding affinity of the IGFBPs to IGF (Rajaram et al., 1997) thus increasing (Elgin et al., 1987) and/or decreasing (Ritvos et al., 1988; Knauer and Smith, 1980) the responses of cells to the IGFs, suggesting that IGFBPs regulate the bioavailability of IGFs to their target tissues (Hardouin et al., 1989). For the most part, IGFBPs function to inhibit IGF action by sequestering free IGF thus preventing it from binding to its receptor (Rechler, 1993). When bound to IGF, the IGFBPs also function to increase the half-life of IGF thus creating large pools of IGFs within the body (Monget et al., 1996; Monget and Bondy, 2000) and maintaining the high levels of IGF in circulation by limiting their transport out of the vasculature (Conover, 1996).

The IGFBPs have also been suggested to act as antigonadotropins by sequestering endogenous IGF, therefore limiting optimal FSH action (for review see Adashi, 1998). Specifically, IGFBP-2, -3, -4 and -5 are potent inhibitors of FSH, but not LH, stimulated steroidogenesis in cultured rat granulosa cells (Ui et al., 1989; Bicsak et al., 1990; Liu et al., 1993). However, IGFBP-3 had no effect on FSH-induced progesterone or estradiol production by culture bovine granulosa cells (Spicer and Chamberlain, 1999). Rather, IGFBP-3 inhibited IGF-I induced progesterone and estradiol production by bovine granulosa cells (Spicer and Chamberlain, 1999). In bovine thecal cells, IGFBP-2 and –3 inhibited IGF-I induced (but not LH induced) progesterone and androstenedione production (Spicer et al., 1997). Conversely, FSH has been found to decrease ovarian expression of the low molecular weight binding proteins in growing healthy

follicles; whereas atretic follicles are characterized by a loss of sensitivity to gonadotropins that allows an increase in expression of the low molecular weight binding proteins (Monget et al., 1996). Specifically, within the granulosa cells of the rat (Liu et al., 1993), FSH substantially inhibits production of the IGFBPs. Similarly, FSH has been found to inhibit bovine granulosa cell IGFBP-2 gene expression (Armstrong et al., 1998; Schams et al., 1999). However, in the mouse FSH had no effect on granulosa cell IGFBP-4 or –5 (Adashi et al., 1997), suggesting species specificity regarding follicular IGFBP production. Inhibition of IGFBP production may serve to increase free IGF resulting in a stimulatory effect on follicular development (for review see Adashi, 1998).

Estradiol also influences intraovarian IGFBP synthesis, but it appears to affect each IGFBP differently. For example, estradiol decreases IGFBP-1 protein and mRNA levels in human luteinizing granulosa cells (Iwashita et al., 1996). Estradiol increases IGFBP-2 mRNA in rat theca-interstitial cells (Ricciarelli et al., 1991), but decreases IGFBP-2 protein synthesis in rat (Ricciarelli et al., 1991) and pig (Mondschein et al., 1990) granulosa cells. Furthermore, IGFBP-2 levels decrease with increases in estradiol concentrations in human (San Roman and Magoffin, 1993), bovine (Echternkamp et al., 1994a; Funston et al., 1996; Stewart et al., 1996), porcine (Howard and Ford, 1992), and ovine (Spicer et al., 1995) follicular fluid. Estradiol does not affect IGFBP-3 or –4 production by porcine granulosa cells (Mondschein et al., 1990). However, diethylstilbesterol (DES) increased IGFBP-4 (Ricciarelli et al., 1991), but decreased IGFBP-6 (Rohan et al., 1993) mRNA in hypophysectomized rats. In comparison, insulin

decreases IGFBP-1 transcription in rats (Orlowski et al., 1991) and humans (Powell et al., 1991), but increases IGFBP-2 (Grimes and Hammond, 1992; Samaras et al., 1993) and –3 (Grimes and Hammond, 1992) production in cultured porcine granulosa cells.

Insulin-like Growth Factor Receptors

The IGFs act by binding to and activating specific membrane bound receptors present on most cells (Conover, 1996). The type I IGF receptor is a $\alpha 2\beta 2$ tetramer, both structurally and functionally related to the insulin receptor (Roth and Kiess, 1994), that mediates most actions of IGF-I and –II through the classical growth factor signaling cascade involving activation of the tyrosine kinase domain of the receptor (LeRoith et al., 1995; Stewart and Rotwein, 1996). It is synthesized on the ribosome as a single polypeptide chain. Post-translational modifications remove a 30-amino acid signal peptide and cleave off the proreceptor into a 706-amino acid extracellular α–subunit and a 626-amino acid transmembrane β-subunit . The α– and β-subunits are linked by disulfide bonds to form a αβ-half-receptor; two αβ-half-receptors are then joined by disulfide bonds to form the mature $\alpha_2\beta_2$ – holoreceptor. The cysteine-rich α-subunit extracellular domain provides the ligand binding specificity, whereas, the cytoplasmic β-domain provides the tyrosine kinase activity (for review see Werner et al., 1991). The cysteine rich area of the type I IGF receptor is necessary for recognition of IGF-I by the receptor (Andersen et al., 1991; Gustafson and Rutter., 1990; Schumacher et al., 1991; Andersen et al., 1992;

Schumacher et al., 1993). The type I IGF receptor binds IGF-II with a 2- to 15-fold lower affinity and insulin with a 100 to 1000-fold lower affinity than for IGF-I (Steel et al., 1988; Germain Lee et al., 1992; LeRoith et al., 1995; Stewart and Rotwein, 1996). The type I IGF receptor and insulin receptor are approximately 50 to 60% homologous overall, with 84% homology among the tyrosine kinase domains (Czech, 1989). The type II IGF, or IGF-II/mannose-6-phosphate (IGF-II/M6P) receptor is a single-chain membrane-spanning receptor (Lucy, 2000) that functions to clear IGF-II from the cell surface, but does not appear to play a role in the classical IGF actions such as mitogenesis (Monget et al., 1996). Unlike the type I receptor, the type II IGF receptor does not act via the classical IGF second messenger pathway (Lucy, 2000) but acts by binding IGF-II, internalizing it via endocytosis, and then degrading it via intracellular lysosomes (Oka et al., 1985; Nolan et al., 1990; Braulke, 1999). The type II/M6P receptor binds IGF-II and molecules with an M6P residue (Nissley and Kiess, 1991) with preferential affinity, binds IGF-I with a 500-fold lower affinity than IGF-II and does not bind insulin (Nissley et al., 1991). Interestingly, the IGF-II binding site is distinctly different than the binding site for mannose 6-phophate (Braulke et al., 1988) and both sites can be occupied simultaneously, however they can interfere with each other (Kiess et al., 1988; Oka et al., 1985; Kiess et al., 1990). Furthermore, the extracellular domain of the type II/M6P IGF receptor can be cleaved by proteases producing a soluable form that may act as a carrier of IGF-II (Clairmont and Czech, 1991).

Within the ovary, IGF receptors and IGF receptor mRNA have been found to exist in porcine (Baranao and Hammond 1984; Otani et al., 1985), bovine (Spicer et al., 1994), rat (Davoren et al., 1986) and human (El-Roeiy et al., 1993; El-Roeiy et al., 1994) granulosa cells, as well as bovine (Stewart et al., 1996), rat (Hernandez et al., 1988a,b) and human (Bergh et al., 1993; El-Roeiy et al., 1993; 1994; Samoto et al., 1993a,b) thecal cells. In addition, bovine (Sauerwein et al., 1992), rat (Ladenheim et al., 1984; Parmer et al., 1991; Talavera and Menon, 1991) and human (Samoto et al., 1993a,b) luteal cells and human stromal cells (Jarrett et al., 1985; Poretsky et al., 1988; Hernandez et al., 1992) contain IGF receptor mRNA and protein.

Type I receptors increase as granulosa cells develop and decrease during atresia (Spicer and Echternkamp, 1995). Specifically, cultured bovine granulosa cells from large follicles had a much greater number of IGF-I receptors than did granulosa cells from small follicles (Spicer et al., 1994) and IGF-I receptor activity increased as follicles grew from preantral to antral size in bovine fetuses and neonatal calves (Wandji et al., 1992). However, numbers of IGF-I receptors in granulosa and thecal cells do not differ between dominant and subordinate bovine follicles (Stewart et al., 1996). In rats, IGF-I receptor mRNA increased in healthy follicles as granulosa cells differentiated and luteinized, but disappeared from granulosa cells of atretic follicles (Zhou et al., 1991). Furthermore, IGF-I binding sites were significantly lower in atretic follicles than in nonatretic follicles of mice (Baker et al., 1996; Adashi et al., 1997; Wandji et al., 1998). In contrast, granulosa cell IGF receptor numbers remained constant among small, medium

and large porcine follicles (Maruo et al., 1988) and type I IGF receptor mRNA did not change as follicles increased from 2 to 8 mm (preovulatory) in diameter in weaned sows (Liu et al., 1999).

Within granulosa cells, type I IGF-I receptors appear to be both gonadotropin and estrogen dependent (for review see Adashi, 1998). Specifically, FSH increases IGF I receptors in granulosa cells from small bovine follicles (Spicer et al., 1994). Furthermore, FSH and LH increases granulosa-cell IGF-I receptor numbers in hypophysectomized DES treated rats (Adashi et al., 1986; Adashi et al., 1988). Estradiol treatment increases IGF-I receptor number in cultured porcine granulosa cells (Veldhuis et al., 1986). Similary, estradiol, but not progesterone, stimulatesIGF-I receptor numbers in bovine granulosa cells (Spicer et al., 1994). However, in vivo, estradiol treatment decreases IGF-I receptor numbers in rat corpora lutea (Parmer et al., 1991). Thus, numbers of granulosa-cell IGF-I receptors are increased by FSH and(or) estradiol in all species evaluated to date.

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CHAPTER III

Effect of Follicle Size on in vitro Production of Steroids and Insulin-like Growth Factor (IGF)-I, IGF-II and the IGF Binding-Proteins (IGFBPs) by Equine Ovarian Granulosa Cells

Abstract

The objectives of this study were to determine 1) if stage of the estrous cycle or follicle diameter influences steroid concentration or components of the IGF system present in follicular fluid of mares (Experiment 1), 2) if estradiol, insulin and/or FSH affect steroid production by equine granulosa cells (Experiment 2A), and 3) if the components of the IGF system are produced by equine granulosa cells in culture, as well as whether estradiol, insulin and/or FSH affects IGF and/or IGFBP production by equine granulosa cells (Experiment 2B). Follicular fluid and granulosa cells from small (<15 mm), medium (16-25 mm) and large (>25 mm) follicles were collected from luteal (n=6) and follicular (n=8) phase mares. Granulosa cells harvested from follicular fluid were cultured 2 days in medium containing 10% fetal calf serum, washed, and then treated for an additional 2 days in serum-free medium with or without added hormones. In Experiment 1, follicular fluid estradiol concentrations differed ($p<0.05$) with estrous cycle stage and follicle diameter. In contrast, progesterone levels were

not affected ($p>0.10$) by either estrous cycle stage or follicle diameter. Concentrations of IGF-I did not differ ($p>0.10$) with estrous cycle stage, but increased with follicle diameter ($p<0.05$). In comparison, IGF-II concentrations differed ($p<0.05$) with estrous cycle stage, but did not differ with follicle diameter ($p>0.10$). IGFBP-2, -3, -4 and –5 appeared in mare follicular fluid, as well as several high molecular weight IGFBPs. Levels of IGFBP-3 tended to differ ($p<0.08$) with estrous cycle stage and increased with follicle diameter during the follicular, but not luteal, phase. The remaining IGFBPs did not differ ($p>0.10$) between phases, but IGFBP-2, -4, and –5 decreased with an increase in follicle diameter ($p<0.05$), while a 125-135 kDa IGFBP tended to decrease with follicle diameter ($p<0.08$). Levels of a 90-96 kDa IGFBP did not change ($p>0.10$) with an increase in follicle diameter. In Experiment 2A, large follicles produced less progesterone than did medium or small follicles ($p<0.05$). Progesterone production was inhibited ($p<0.05$) by FSH and insulin in small and medium but not large follicles; estradiol was without effect. Insulin increased ($p<0.05$) estradiol production in small and medium follicle granulosa cells but had no effect in large follicle granulosa cells. In Experiment 2B, IGF-I production was inhibited ($p<0.05$) by insulin across all follicle sizes, but was not affected by estradiol or FSH. Granulosa cells of medium and large follicles produced more IGF-II than did granulosa cells of small follicles ($p<0.05$). Insulin and FSH inhibited ($p<0.05$) IGF-II production by granulosa cells of large and medium but not small follicles; estradiol was without effect. Of the four IGFBPs present in follicular fluid of mares, IGFBP-2 and –5 were the only ones produced by equine granulosa cells.

IGFBP-2 production was less ($p<0.10$) in granulosa cells of large vs small and medium follicles. Averaged across follicle sizes, estradiol increased ($p<0.10$) IGFBP-2 production, whereas FSH increased ($p<0.10$) IGFBP-2 and -5 production; insulin was without effect. These results indicate that IGF-I, IGF-II, IGFBP-2, -4 and –5 are present in equine follicle fluid and that estrous cycle stage and follicle diameter influence concentrations of steroids and components of the IGF system. Furthermore, these results indicate that IGF-I, IGF-II, IGFBP-2 and –5 are produced by equine granulosa cells and that insulin, FSH and estradiol play a role in the regulation of steroidogenesis and the IGF system of equine granulosa cells.

Introduction

The insulin-like growth factor (IGF) system, composed of IGF-I, IGF-II, IGF receptors and IGF-binding proteins (IGFBPs), plays an essential role in ovarian function (for reviews see Sara and Hall, 1990; Hammond et al., 1991; Giudice, 1992; Monget and Monniaux, 1995; Spicer and Echternkamp, 1995). In most species, IGF-I stimulates granulosa and thecal cell proliferation and mitogenesis, and synergizes with gonadotropins to stimulate granulosa and thecal cell steroidogenesis (for reviews see Hammond et al., 1991; Giudice, 1992; Monniaux and Pisselet, 1992).

The IGFs are found in the systemic circulation bound to high affinity, soluble, carrier proteins (for reviews see Baxter, 1988; Clemmons, 1993; Rechler, 1993; Spicer and Echternkamp, 1995). These binding proteins have a

greater affinity for the IGFs compared to IGF receptors and, therefore, are thought to act as regulators of IGF availability for target cells (Baxter, 1988; Clemmons, 1993). The presence of IGFBP mRNA in ovarian tissue was first reported in 1989 (Margot et al, 1989; Shimasaki et al, 1989; Ui et al, 1989) and since that time additional IGFBPs (i.e., IGFBP-1 through - 8) have been identified in the ovary of several species (for reviews see Rechler, 1993; Spicer and Echternkamp, 1995). Within the follicle, IGFBP-3 levels do not change during development, yet it is the most predominant IGFBP found in ovarian follicular fluid of pigs (Mondschein et al., 1991; Echternkamp et al., 1994b), cattle (Echternkamp et al., 1994a; Stewart et al., 1996), sheep (Spicer et al., 1995) and horses (Gerard and Monget, 1998). In contrast, follicular fluid IGFBP-2, -4 and -5 activity is lower in growing (estrogen active) dominant vs. subordinate (estrogen inactive) follicles in cattle (Echternkamp et al., 1994a; Stewart et al., 1996), pigs (Grimes et al, 1994b), sheep (Monget et al., 1993) and horses (Gerard and Monget, 1998). These studies suggest that follicular fluid IGFBP-2, -4 and -5 levels are closely related to the physiological status of follicles in several species. Some of these IGFBPs are produced within the follicle by granulosa cells of pigs and rats (for review see Spicer and Echternkamp, 1995). However, which IGFBPs are produced by granulosa cells of the mare is unknown.

In the mare, one follicle is selected from a cohort of follicles to become dominant. After selection the dominant follicle continues to grow until ovulation, while the remaining cohort, or subordinate, follicles become atretic and regress (for review see Ginther, 1992). Limited information is available regarding the

physiological mechanism of follicle selection and maturation in the mare. During preovulatory development in the mare, follicular fluid IGF-I levels increase (Spicer et al., 1991) whereas IGFBP levels decrease (Gerard and Monget, 1998). In cattle, it has been hypothesized that estradiol and the gonadotropins induce changes in the amount of IGF-I and IGFBPs produced by granulosa and thecal cells during follicular development (Spicer et al., 1988; Spicer and Enright, 1991; Stewart et al., 1996). We hypothesize that, in the mare, follicular development may involve steroid or gonadotropin induced changes in the intrafollicular IGF system.

Therefore, the specific objectives of these experiments were to 1) determine whether stage of the estrous cycle or follicle diameter influences concentrations of steroids, as well as components of the IGF system, present in follicular fluid of mares, 2) determine whether estradiol, insulin and/or FSH affect steroid production by equine granulosa cells, and 3) determine what components of the IGF system are produced by equine granulosa cells in culture, as well as to determine whether estradiol, insulin and/or FSH affect IGF and/or IGFBP production by equine granulosa cells.

Materials and Methods

Reagents and Hormones

The reagents used were as follows: Dulbecco's modified Eagle medium (DMEM), Ham's F-12, sodium bicarbonate, gentamicin, insulin (bovine; 28.5 U/mg), trypan blue, fetal calf serum (FCS), estradiol and acrylamide, all obtained

from Sigma Chemical Company (St, Louis, MO.); ovine FSH (F1913; FSH activity 15 x NIH-LH-S1 U/mg) obtained from Scripps Laboratories (San Diego, CA); nitrocellulose transfer membrane (.45 μm pore size) obtained from Midwest Scientific (St. Louis, MO); protease-free bovine serum albumin (BSA) obtained from Integrin (Purchase, NY); 20% (w/v) sodium dodecyl sulfate solution (SDS) obtained from Amresco (Solon, OH); recombinant bovine IGF-II obtained from Monsanto (St. Louis, MO).

Cell Culture

In late May, 1988, a total of 28 ovaries were obtained at a commercial abattoir from 14 mares of various breeds, ages and sexual maturity. The mares were classified as either in the luteal (n = 6) or follicular (n = 8) phase 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 large follicles and a corpus albicans or regressing CL (as indicated by pale color and little or no vascularity with a diameter < 20 mm) were classified as being in the follicular phase (Ireland et al., 1980). The ovaries were processed at the abattoir (and in transit from the abattoir) as previously described for bovine follicles (Langhout et al., 1991; Stewart et al., 1995). Follicular fluid from individual follicles were collected separately using needles and syringes. Specifically, follicular fluid was aspirated from the follicle. Without removing the needle from the follicle, follicular fluid was injected back into the follicle and aspirated out again while the follicle was hand massaged to loosen granulosa cells. This was

repeated two times per follicle. After granulosa cells were separated from follicular fluid by centrifugation (220 X g for 5 to 7 min), individual follicular fluid samples were frozen at –80 °C. Follicles were separated into three groups based on surface diameter: small (6-15 mm), medium (16-25 mm) and large (25-48 mm) (Driancourt and Palmer, 1984). Granulosa cells from individual follicles were combined into three pools within each of the three size categories without regard to phase of cycle, washed twice in serum-free medium by centrifugation at 200 X g (for 5 to 7 min) and resuspended in medium containing 1 mg/mL collagenase and 0.01 mg/mL DNase to disperse and prevent clumping of the cells. The number and viability granulosa cells was determined using a hemocytometer and the trypan blue exclusion method, and averaged 74.9 ± 8.9%, 79.2 ± 5.5% and 79.9 ± 10.98% of total granulosa cells, respectively from small, medium and large follicles.

Medium consisted of a 1:1 (vol/vol) mixture of DMEM and Ham's F-12 containing 0.12 mM gentamicin, 20 mM glutamine and 38.5 mM sodium bicarbonate. Approximately 4 X 10^5 viable cells in 100 μL of medium were added to Falcon 24-well plates (No. 3047; Becton Dickinson and Co., Lincoln Park, NJ) containing 1 mL of medium with 10% FCS. Cultures were kept at 38.5°C in a 95% air-5% CO_2 atmosphere (Langhout et al., 1991). Cell proliferation was monitored daily using phase contrast microscopy. To obtain optimal attachment, cells were maintained in 10% FCS without added hormones for the first 2 days of culture. After 48 h, cells were washed twice with 0.5 mL of serum-free medium to remove FCS and non-adherent cells, and incubations continued in serum-free

medium (0.5 mL) containing 500 ng/mL of testosterone (as an estradiol precursor) and 2.5 mg/mL of BSA (to minimize loss of the IGFBPs) with or without added hormones for an additional 48 h. Throughout the 4-day culture, medium was changed every 24 h.

Experiment 1 was designed to determine whether stage of the estrous cycle or follicle diameter influenced concentrations of steroids and components of the IGF system in mare follicular fluid. Follicular fluid was aspirated from follicles as described earlier and assayed for concentrations of estradiol, progesterone, androstenedione, IGF-I and IGF-II (see section on radioimmunoassays). Also, follicular fluid was assessed for IGFBP activity based on molecular weight using one dimensional, reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (see later section on gel electrophoresis).

Experiment 2A was designed to determine whether estradiol, insulin and/or FSH affected steroid production by equine granulosa cells. Granulosa cells from small (6-15 mm), medium (16-25 mm) and large (25-48 mm) follicles were cultured in medium containing 10% FCS for 48 h as described earlier. After 48 h, the media was replaced with serum-free medium containing testosterone and either no hormone addition (control), estradiol (500 ng/mL), insulin (100 ng/mL), FSH (50 ng/mL), insulin (100 ng/mL) plus estradiol (500 ng/mL), or insulin (100 ng/mL) plus FSH (50 ng/mL) and incubated for an additional 48 h. At the end of the first 24 h incubation with treatments (i.e., during day 2 to 3 of culture) medium was collected for estradiol and progesterone measurement and fresh medium was added.

Experiment 2B was designed to determine what components of the IGF system are produced by equine granulosa cells in culture, as well as to determine whether estradiol, insulin and/or FSH affected IGF and/or IGFBP production by granulosa cells. Granulosa cells from small (6-15 mm), medium (16-25 mm) and large (25-48 mm) follicles were cultured as described in Experiment 2A. Treatments were no additions (control), estradiol (500 ng/mL), insulin (100 ng/mL), FSH (50 ng/mL), insulin (100 ng/mL) plus estradiol (500 ng/mL), or insulin (100 ng/mL) plus FSH (50 ng/mL). At the end of the second 24 hour incubation with treatments (i.e., during day 3 to 4 of culture) medium was collected for IGF and IGFBP assessment.

Determination of Cell Numbers

After the treatment period had elapsed, medium from each well was collected individually and frozen at (- 20 °C) for later use. Numbers of granulosa cells were determined at the termination of experiments (i.e., day 4 of culture) using a Coulter counter (Model Zm; Coulter Electronics, Hialeah, FL) as previously described (Baranao and Hammond, 1985; Langhout et al., 1991). Briefly, cells were exposed to 0.5 mL of trypsin (0.25%[wt/vol] in 0.15 M NaCl) for 20 min at 25°C, then scraped from each well with a teflon policeman, diluted in 0.15 M NaCl and enumerated.

Concentration of Spent Medium

Media samples collected for IGF and IGFBP assessment were ultrafiltrated using Centricon concentrators with a molecular weight (MW) limit of 3,000 (Amicon, Inc., Beverly, MA). The spent medium was concentrated 5- to 18-fold. Briefly, 400 μL of the spent media was placed inside the sample reservoir of the concentrator and centrifuged at 5322 X g for approximately 80 min. After centrifugation, the filtrate vial was discarded, the sample reservoir was inverted and re-centrifuged for 5 min at 591 X g to transfer the retentate into the retentate vial. Final volumes ranging from 22 to 77μL were measured to record the concentration factor of the sample; this value was used to correct IGF and IGFBP data.

Protein Determination

Amount of protein in follicular fluid was quantified as previously described (Lowry et al., 1951). Assay sensitivity (i.e., the lowest value at 95% confidence interval of the lowest point on the standard curve) was 1.79 μg/tube and the intra-assay coefficient of variation was 15.0%.

Radioimmunoassays (RIA)

Progesterone RIA. Concentrations of progesterone in follicular fluid and in culture medium collected 24 h after hormone treatments were determined with a double-antibody RIA as previously described (Baranao and Hammond, 1985; Langhout et al., 1991). Assay sensitivity (i.e., 95% of total binding) was 0.016

±0.007 ng/tube and intra- and interassay coefficients of variation were 12.9 and 17.5%, respectively.

Estradiol RIA. Concentrations of estradiol-17β in follicular fluid and in culture medium collected 24 h after hormone treatments were determined with a double-antibody RIA as previously described (Spicer and Enright, 1991; Spicer and Stewart, 1996). Assay sensitivity (i.e., 90% of total binding) was 0.29 ± 0.05 pg/tube, and the intra- and interassay coefficients of variation were 12.5 and 10.4%, respectively.

Androstenedione RIA. Concentrations of androstenedione in follicular fluid were determined using solid-phase RIA kits (ICN Biomedicals, Costa Mesa, CA) as previously described (Stewart et al., 1995). Assay sensitivity (i.e., 95% of total binding) was 1.45 ± 0.36 pg/tube and the intra- and interassay coefficients of variation were 15.0 and 17.0%, respectively.

IGF-I RIA. Concentrations of IGF-I in follicular fluid and in concentrated culture medium collected 48 h after hormone treatments were determined with a double-antibody RIA after acid-ethanol extraction (16 h at 4°C) as previously described (Echternkamp et al., 1990; Spicer et al., 1991). Assay sensitivity (i.e., 95% of total binding) was 4.89 ±0.72 ng/tube and the intra- and interassay coefficient of variation was 14.5 and 18.8%, respectively.

IGF-II RIA. Concentrations of IGF-II in follicular fluid and in concentrated culture medium collected 48 h after hormone treatments were determined with a double-antibody RIA as previously described for bovine and ovine follicular fluid (Spicer et al., 1995; Stewart et al., 1996) and validated for equine follicular fluid.

Increasing volumes of extracted equine follicular fluid as well as concentrated spent culture medium from equine granulosa cell cultures created displacement curves parallel to the standard curve. Assay sensitivity (i.e., 95% of total binding) was 22.98 ± 9.40 pg/tube, and intraassay coefficient of variation was 9.17%.

Gel Electrophoresis of IGFBPs

IGFBPs in follicular fluid and concentrated culture medium collected 48 h after hormone treatment were analyzed by one-dimensional, reducing, SDS-PAGE based on molecular weight as previously described (Echternkamp et al., 1994a; Simpson et al., 1997). Briefly, for culture medium, 12.5 μL of concentrated sample was mixed with 12.5 μL of Laemmli sample buffer (BIORAD, Hercules, CA). For follicular fluid, 4 μL of sample was mixed with 21 μL of Laemmli sample buffer (BIORAD, Hercules, CA). The samples were heat denatured (3 min at 100°C), centrifuged at 4657 X g for 3 min and then electrophoresed on a 12% polyacrylamide gel overnight (approximately 18 to 20 h) at a constant current (25 to 35 amps) and varying voltage. After separation, proteins in gels were electrophoretically transferred to nitrocellulose paper (Midwest Scientific, St. Louis, MO) for 2.5 to 3.0 h. Each nitrocellulose paper was incubated with 6 mL of ^{125}I-IGF-II (15,000 cpm/100 μL) and placed on a rocking platform at 4°C overnight. The next day, the blots were washed in a Tris-buffered saline (TBS) with 0.1% Tween followed with additional washings with only the TBS. The nitrocellulose blots were then dried and exposed to X-ray film at (-80 °C) for 12 days for follicular fluid and 4 days for culture medium. Individual band

intensity on autoradiographs was densitometrically analyzed using a Molecular Analyst (BIORAD, Hercules, CA).

Statistical Analysis

Data were analyzed using PROC MIXED of SAS. For Experiment 1, linear regressions were performed for each response variable (E_2, P_4, A_4, protein, IGF-I, IGF-II and IGFBPs) using the variable "SIZE" as the independent variable. Separate regressions were performed for each stage (Luteal or Follicular). Dummy variables for each response were used to assess whether the slopes associated with each stage were significantly different. If the slopes associated with the two stages were not statistically significant, then a model to assess the difference in the intercepts for the two stages was performed. For Experiment 2A and 2B interaction of size by treatment was assessed. In the absence of interaction, orthogonal contrasts to assess the main effects of estradiol, FSH and insulin were utilized. In the presence of interaction, contrasts were formed to evaluate the simple effects of estradiol, FSH and insulin on each follicle size. Multiple mean comparisons (LSD) were performed only if a main effect was significant. For Experiment 2B, correction factors were utilized to standardize volume of concentrated media.

Results

Experiment 1: Effect of stage of estrous cycle and follicle diameter on steroid concentrations and the IGF system in equine follicular fluid.

Estradiol production differed during the follicular phase as compared to the luteal phase ($p<0.05$). Fitted lines for both the follicular and luteal phases are shown in Figure 1. During the follicular phase, estradiol concentration increased with an increase in follicle diameter ($p<0.05$). In contrast, diameter appeared to play little or no role in increasing estradiol concentration during the luteal phase ($p>0.10$). In contrast to estradiol, progesterone concentration in follicular fluid averaged 28.9 ± 3.8 ng/mL and did not differ ($p>0.10$) between the follicular and luteal phase, nor was it affected ($p>0.10$) by follicle diameter (data not shown).

Androstenedione concentration in follicular fluid averaged 80.09 ± 28.43 ng/mL and did not differ ($p>0.10$) between the follicular and luteal phase. However, androstenedione concentration increased as follicle diameter increased ($p<0.05$). The fitted line for the combined follicular and luteal phases of androstenedione are shown in Figure 2.

The concentration of protein found in follicles of the follicular phase was different ($p<0.05$) than that seen in luteal phase follicles. During both phases of the estrous cycle, protein concentrations increased with an increase in follicle diameter ($p<0.05$; data not shown).

Concentrations of IGF-I in follicular fluid during the follicular phase (45.9 ± 4.7 ng/mL) did not differ ($p>0.10$) from those during the luteal phase (34.1 ± 5.6 ng/mL). However, IGF-I concentration increased as follicle diameter increased ($p<0.05$). The fitted line for the combined follicular and luteal phases of IGF-I are shown in Figure 3. Unlike IGF-I, IGF-II concentrations were different ($p<0.05$) between the follicular (61.4 ± 3.6 ng/mL) and luteal (51.3 ± 4.3 ng/mL) phase, but

IGF-II concentrations remained similar ($p>0.10$) as follicle diameter increased (data not shown).

Of the known IGFBPs, IGFBP-2, -3, -4 and -5 were present in mare follicular fluid (Figure 4). We also detected several IGFBPs with estimated molecular weights ranging between 90 and 135 kDa. Levels of IGFBP-3 tended to differ ($p<0.08$) between the follicular and luteal phases and increased ($p<0.05$) with an increase in follicle diameter during the follicular stage but not the luteal phase. The fitted lines for IGFBP-3 of both follicular and luteal phases are shown in Figure 5. In contrast, the other IGFBPs did not differ ($p>0.10$) between phases (data not shown). IGFBP-2, -4 and -5 decreased ($p<0.05$) with an increase in follicle diameter (Figure 6). The 125-135 kDa IGFBP and the 115 kDa IGFBP tended to decrease ($p<0.08$) with an increase in follicle diameter (data not shown). Concentrations of the 90-96 kDa IGFBP did not change ($p>0.10$) with follicle diameter (data not shown).

Experiment 2A: Effect of insulin, FSH and estradiol on granulosa cell steroid production

Basal levels of progesterone production were lower ($p<0.05$) in large- versus small- and medium-follicle granulosa cells (Figure 7). Progesterone production was inhibited ($p<0.05$) by insulin, FSH, and a combination of insulin plus FSH in cultures of small- and medium- but not large-follicle granulosa cells. Estradiol alone had no effect ($p>0.10$) on progesterone production in medium- and large-follicle granulosa cells, but inhibited ($p<0.05$) progesterone production

in small-follicle granulosa cells. When estradiol was combined with insulin, progesterone production was inhibited ($p<0.05$) in cultures of small- and medium- but not large-follicle granulosa cells; this inhibition was similar to that seen with insulin alone (Figure 7).

Basal levels of estradiol production did not differ ($p>0.05$) in cultures of small-, medium- or large-follicle granulosa cells (Figure 8). Insulin stimulated ($p<0.05$) estradiol production by small- and medium-follicle granulosa cells, but was without effect ($p>0.10$) in large follicles. FSH treatment alone had no effect ($p>0.10$) on estradiol production by small- and large-follicle granulosa cells, but inhibited ($p<0.05$) estradiol production by 90% in medium follicles. FSH also inhibited ($p<0.05$) the insulin-induced increase in estradiol production by medium follicles. In small follicles, the combined treatment of insulin and FSH stimulated ($p<0.05$) estradiol production 5-fold compared to controls and 8-fold compared to FSH treated cultures; granulosa cells of large follicles were not affected ($p>0.10$) by the insulin and FSH combination (Figure 8).

Experiment 2B: Effect of insulin, FSH and estradiol on granulosa cell IGF and IGFBP production

Equine granulosa cells produced 10- to 50-fold more IGF-II (Figure 9) than IGF-I (Table 1) in culture. When averaged across follicle size, insulin inhibited ($p<0.05$) IGF-I production 24% to 36%, but was not affected by estradiol or FSH (Table 1). In comparison, singular treatments of insulin or FSH inhibited ($p<0.05$) IGF-II production by medium- and large- but not small-follicle granulosa cells

(Figure 9). Combined treatments of insulin plus FSH also inhibited ($p<0.05$) IGF-II production by medium- and large- but not small-follicle granulosa cells; this inhibition by combined treatments did not differ ($p>0.10$) from either singular treatment. Estradiol alone inhibited ($p<0.05$) IGF-II production by large-follicle granulosa cells but had no effect in small or medium follicles. Medium- and large-follicle granulosa cells produced more ($p<0.05$) basal IGF-II than did small-follicle granulosa cells (Figure 9).

Equine granulosa cells produced two forms of IGFBP: a 29-40 kDa IGFBP (IGFBP-2) and a 29-31 kDa IGFBP (IGFBP-5; Figures 10, 11 and 12). Size of follicle did not influence ($p>0.10$) production of IGFBP-5; however IGFBP-2 production by granulosa cells of large follicles tended ($p<0.10$) to be 35% to 59% less than small and medium follicles. Treatment tended ($p<0.10$) to affect both IGFBP-2 and IGFBP-5 production (Table 1). When averaged across follicle size, estradiol increased ($p<0.10$) only IGFBP-2 production, whereas FSH increased ($p<0.10$) both IGFBP-2 and IGFBP-5 production. Insulin alone was without effect ($p>0.10$) on IGFBP-5 production, but tended ($p<0.06$) to increase IGFBP-2 production in large follicles and decrease IGFBP-2 production in medium and small follicles (data not shown). Insulin combined with either estradiol or FSH blocked ($p<0.10$) the stimulatory effect of estradiol on production of IGFBP-2 and the stimulatory effect of FSH on production of IGFBP-5 (Table 1).

Representative examples of cultured equine granulosa cells from small, medium and large follicles are depicted in Figures 10, 11 and 12. Insulin stimulated ($p<0.05$) granulosa cell numbers in cultures from small and medium

follicles, but was without effect in cultures from large follicles (Figure 13). Estradiol and FSH treatments alone had no effect ($p>0.10$) on granulosa cell numbers in cultures from small, medium or large follicles. When estradiol or FSH was combined with insulin, granulosa cell numbers increased ($p<0.05$); these increases were similar to those seen with insulin alone (Figure 13).

Discussion

Results of the present study revealed that 1) follicular fluid levels of IGFBP-2, -4 and –5 decreased with follicular size whereas levels of estradiol and IGFBP-3 increased with follicular size, 2) of the IGFBPs found in follicular fluid only IGFBP-2 and -5 are produced by equine granulosa cells, 3) FSH and estradiol increased IGFBP-2 and –5 production, had no effect on IGF-I production, and decreased IGF-II production by equine granulosa cells, and 4) estradiol and progesterone production by equine granulosa cells were differentially regulated by insulin and FSH and these effects were dependent on follicle size.

In the present study, intrafollicular levels of the low molecular weight IGFBPs (IGFBP-2, -4 and –5) decreased as follicle diameter increased regardless of estrous cycle stage in the mare. This result is consistent with those obtained from cyclic pony mares in which IGFBP-2 binding activity decreased during growth of the dominant follicle but was substantially increased in subordinate cohort follicles (Gerard and Monget, 1998). Similarly, intrafollicular levels of IGFBP-2 and –4 in pigs (Yuan et al., 1996) and IGFBP-2, -4 and –5 in

sheep (Monget et al., 1993; Spicer et al., 1995) and cattle (Echternkamp et al., 1994a; Stewart et al., 1996) significantly decrease as follicle diameter increases probably due to a decrease in gene expression (Samaras et al., 1992; Besnard et al., 1996a,b; Armstrong et al., 1998), an increase in proteolytic degradation of the binding proteins (Besnard et al., 1996b; Besnard et al., 1997) or both. Unlike the low molecular weight IGFBPs, we found that IGFBP-3 levels were unchanged as follicle size increased during the follicular phase but increased with an increase in follicle size during the luteal phase. Increases in follicular fluid IGFBP-3 with increased follicle size have been observed in some studies with sheep (Monget et al., 1993) and pigs (Grimes et al., 1994b). However, other studies report that IGFBP-3 does not change with follicle size in pigs (Howard and Ford, 1992; Echternkamp et al., 1994b), humans (Cataldo and Giudice, 1992), sheep (Monget et al., 1993; Spicer et al., 1995) and cattle (Echternkamp et al., 1994a; Funston et al., 1996; Stewart et al., 1996). Gerard and Monget (1998) found that follicular fluid IGFBP-3 content does not change in equine follicles ranging from 22 mm in diameter to preovulatory size (> 35 mm). This latter observation is not inconsistent with the present study because the lowest levels of IGFBP-3 were found in follicles < 10 mm (Figure 3).

Previous findings are consistent with our observations that intrafollicular estradiol increases during follicular growth (Meinecke et al., 1987; King and Evans, 1988; Sirois et al., 1990; Gerard et al., 1998; Gerard and Monget, 1998; Goudet et al., 1999). Van Rensburg and Van Niererk (1968), using 16 follicles, noted increases in follicular fluid estradiol concentrations with increases in

diameter of follicles > 20 mm. Similarly, our results indicate that concentrations of estradiol do not dramatically increase until follicles reach > 25 mm in diameter. Gerard and Monget (1998) indicated that an increase in estradiol levels occur from early dominant (22-25 mm) follicles to late dominant (33-35 mm) follicles. Similarly, Belin and coworkers (2000) found that estradiol levels were lower in dominant follicles at emergence (approximately 20 mm in diameter) than at the end of follicular growth (≥ 30 mm in diameter) or in preovulatory follicles. Aromatase activity dramatically increases from 20-24 mm follicles to 25-29 mm follicles at the end of the follicular phase (Goudet et al., 1999). Collectively, the past and present research suggests that as follicles develop and become estrogen active, IGFBP-2, -4, and –5 decrease whereas IGFBP-3 remains constant or increases creating a total net decrease in IGFBP activity. In the mare, this decrease in net IGFBP activity concomitant with an increase in total IGF-I levels may act to increase the amount of free IGF-I within the follicle and subsequently increase the sensitivity of the follicle to gonadotropins, stimulating granulosa cell differentiation and follicular development. Those follicles showing increased levels of the low molecular weight IGFBPs and decreased estradiol may be destined for atresia (Giudice, 1992; Monget and Monniaux, 1995; Armstrong et al., 1996; Monget et al., 1996).

For the first time, IGFBP production by equine granulosa cells has been evaluated. Of the IGFBPs detected in equine follicular fluid (IGFBP-2, -3, -4 and –5), only IGFBP-2 and –5 were produced by equine granulosa cells, and both were responsive to hormone treatment. Previous research suggests

that granulosa cell production of IGFBPs is highly species specific. For example, a 29 kDa (presumably IGFBP-1) is produced by ovine granulosa cells, as well as IGFBP-2 and –5 (Armstrong et al., 1996). Granulosa cells from mice (Adashi et al., 1997) and cattle (Chamberlain and Spicer, 1998) produce IGFBP-2, -4 and –5 and porcine granulosa cells produce IGFBP-2, -4 and –5, as well as IGFBP-3 (Grimes et al., 1994a). In the present study, we determined that follicle size had no influence on IGFBP-5 production, but IGFBP-2 production tended to be 35% to 59% less in large- vs. small- and medium-follicle granulosa cells. The present study also revealed that FSH and estradiol treatments increased IGFBP-2 and –5 production by equine granulosa cells. These results are in contrast with studies in pigs (Mondschein et al., 1990; Grimes et al., 1992) that found FSH to be one of the most potent inhibitors of IGFBP-2. Simarlily, FSH has been observed to inhibit granulosa cell IGFBP-2 production in rats (Bicsak et al., 1990) and IGFBP-2 mRNA in cattle (Armstrong et al., 1998; Schams et al., 1999) but did not effect IGFBP-2 production in human luteinizing granulosa cells (Cataldo et al., 1993) or ovine granulosa cells (Monget et al., 1998). In comparison, estradiol inhibits granulosa cell IGFBP-2 production in rats (Ricciarelli et al., 1991) and pigs (Mondschein et al., 1990), but increases plasma IGFBP-5 levels in cattle (Simpson et al., 1997). In the mare, granulosa cell production of IGFBP-2 and –5 may play an inhibitory role on IGF-I action in follicular cells as reported in other species (Liu et al., 1993; Spicer et al., 1997; Spicer and Chamberlain, 1999). High expression of these binding proteins within a follicle may limit free IGF-I, thus slowing follicular growth and allowing emergence of a dominant follicle

(Stewart et al., 1996; Adashi, 1998). Therefore, inhibition of intrafollicular production of IGFBP-2 and –5 may be necessary, at least in part, for selection of the dominant follicle in the mare.

Similar to what was observed in the present study, follicular fluid levels of IGFBP-2 decrease with increases in estradiol concentrations in humans (San Roman and Magoffin, 1993), cattle (Echternkamp et al., 1994a; Funston et al., 1996; Stewart et al., 1996), pigs (Howard and Ford, 1992), and sheep (Spicer et al., 1995). Therefore, it is likely that follicular fluid levels of IGFBP-2, -4 and –5 are initially high at follicular emergence due to increases granulosa cell production in response to high levels of FSH in circulation. When follicles reach approximately 20 mm in diameter, just prior to selection of the dominant follicle in the mare (for review see Ginther, 2000), follicular fluid levels of IGFBP-4 and –5 begin to decrease most probably due to actions of specific IGFBP proteases concomitant with the increase in intrafollicular estradiol and the decline in FSH. In response to declining FSH levels, granulosa cell production of IGFBP-2 and –5 slows. Once the dominant follicle has been selected, estradiol levels increase, further stimulating granulosa cell IGFBP-2 and –5 production in atretic, cohort follicles.

For the first time, hormonal regulation of equine granulosa cell derived IGFs was observed. The present study revealed that FSH and estradiol treatment had no effect on IGF-I production but decreased IGF-II production by equine granulosa cells. Also, insulin and FSH inhibited IGF-II production by medium- and large- but not small-follicle granulosa cells, suggesting that IGF-II

production by small-follicle granulosa cells is unresponsive to trophic factors in the mare. In comparison to our findings, FSH treatment has no effect on IGF-I production by bovine granulosa cells (Spicer et al., 1993; Spicer and Chamberlain, 2000a), but stimulates IGF-I production by porcine granulosa cells (Hsu and Hammond, 1987a,b; Mondschein et al., 1988). In humans, FSH stimulates IGF-II, but not IGF-I, production and mRNA by granulosa cells (Ramasharma and Li, 1987). In bovine granulosa cells, estradiol enhances the insulin-induced decreases in IGF-I and IGF-II production but has no effect on basal IGF-I and IGF-II production (Spicer and Chamberlain, 2000a,b). However, in cultured ovine (Wathes et al., 1995) and porcine (Hsu and Hammond, 1987b) granulosa cells, estradiol increases IGF-I production and in vivo treatment of immature hypophesctomized rats with DES increases IGF-I mRNA, but decreases IGF-II mRNA within the ovary (Hernandez et al., 1990). Thus, species differences may exist in regard to hormonal control of ovarian IGF production.

The present study also revealed that estradiol and progesterone production by equine granulosa cells were differentially regulated by insulin and FSH. We found that insulin decreased progesterone production but increased estradiol production in small- and medium-, but not large-follicle granulosa cells. Similarly, insulin stimulates estradiol production by small-follicle bovine granulosa cells (Spicer et al., 1993; Spicer et al., 1994; Spicer and Chamberlain, 1998), but has no effect or decreases estradiol production in pigs (small vs large follicles; Veldhuis et al., 1983; Maruo et al., 1988), and humans (Erickson et al., 1990). However, insulin and FSH stimulates progesterone production by granulosa cells

of cattle (Schams et al., 1988; Spicer et al., 1993), swine (Baranao and Hammond, 1984), and rats (Davoren et al., 1986). We also observed that FSH alone decreased progesterone production by small- and medium-, but had no effect on large-follicle granulosa cells. Why insulin and FSH are inhibitory to progesterone production in mares but stimulatory in other species remains to be elucidated, but may involve some species specific mechanism unique to the mare. FSH alone did not affect estradiol production by small- and large-follicle granulosa cells, but decreased estradiol production by 90% in medium-follicle granulosa cells. However, in the presence of insulin, FSH enhanced the stimulatory effect of insulin on estradiol production by small follicle granulosa cells, and inhibited the stimulatory effect of insulin on estradiol production by medium-follicle granulosa cells. Sirois et al. (1991) reported that in the presence of insulin, FSH was unable to stimulate estradiol production in large equine follicles during the early estrous phase but was able to significantly stimulate progesterone production by granulosa cells from large (early and late estrus stage) follicles. Also, equine granulosa cell responsiveness to FSH in terms of estradiol and progesterone production decreased as follicle diameter increased in early to late estrus follicles (39 to 47 mm; Sirois et al., 1990), which is constant with results of the present study. Therefore, high circulating levels of FSH, as seen during early follicular growth, may act to inhibit premature granulosa cell differentiation and/or luteinization in small and medium follicles as indicated by the decreased levels of progesterone and estradiol in the present study. Once follicles reach a large diameter (> 25 mm) FSH receptors may be downregulated

making these follicles unresponsive to FSH. Further research is needed to understand hormonal regulation of granulosa cell steroidogenesis in the mare.

The present study indicates that IGF-I, IGF-II, IGFBP-2, -4 and –5 are present in equine follicle fluid and that estrous cycle stage and follicle diameter influence concentrations of steroids and components of the IGF system. Furthermore, these results indicate that IGF-I, IGF-II, IGFBP-2 and –5 are produced by equine granulosa cells and that insulin, FSH and estradiol play a role in the regulation of steroidogenesis and the IGF system of equine granulosa cells. The present study suggests that decreased levels of low molecular weight IGFBPs in large estrogen-active follicles may allow an increase in free IGF-I and thus increase gonadotropin responsiveness and growth of the future dominant follicle. In contrast, increased levels of the low molecular weight IGFBPs in small cohort follicles may act to sequester IGF-I causing a low responsiveness to IGF-I and FSH, thus slowing growth rate of future subordinate follicles.

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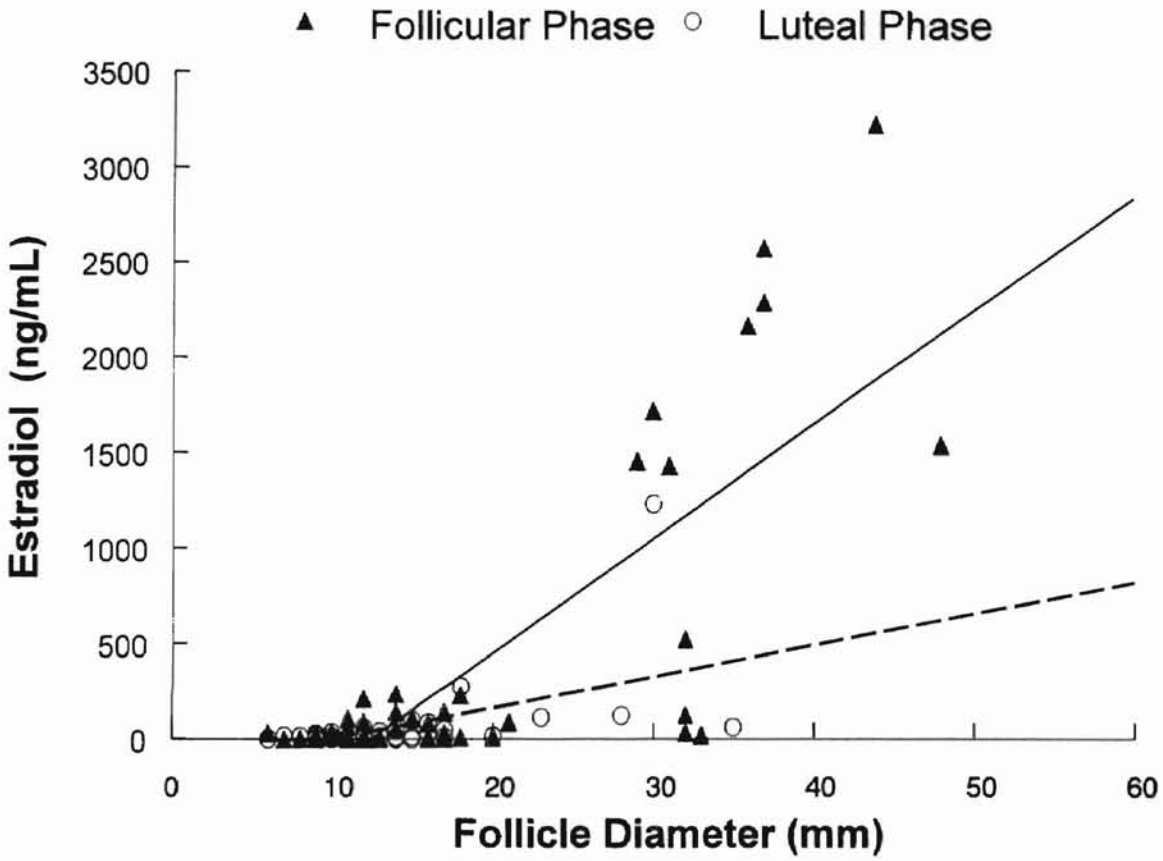

Figure 1. Linear regression analysis of estradiol concentration in follicular fluid. Estradiol concentration in follicles during the follicular phase (——) is represented by the line y = 59.62x - 740.71. Estradiol concentration in follicles during the luteal phase (– – –) is represented by the line y = 16.40x – 163.21.

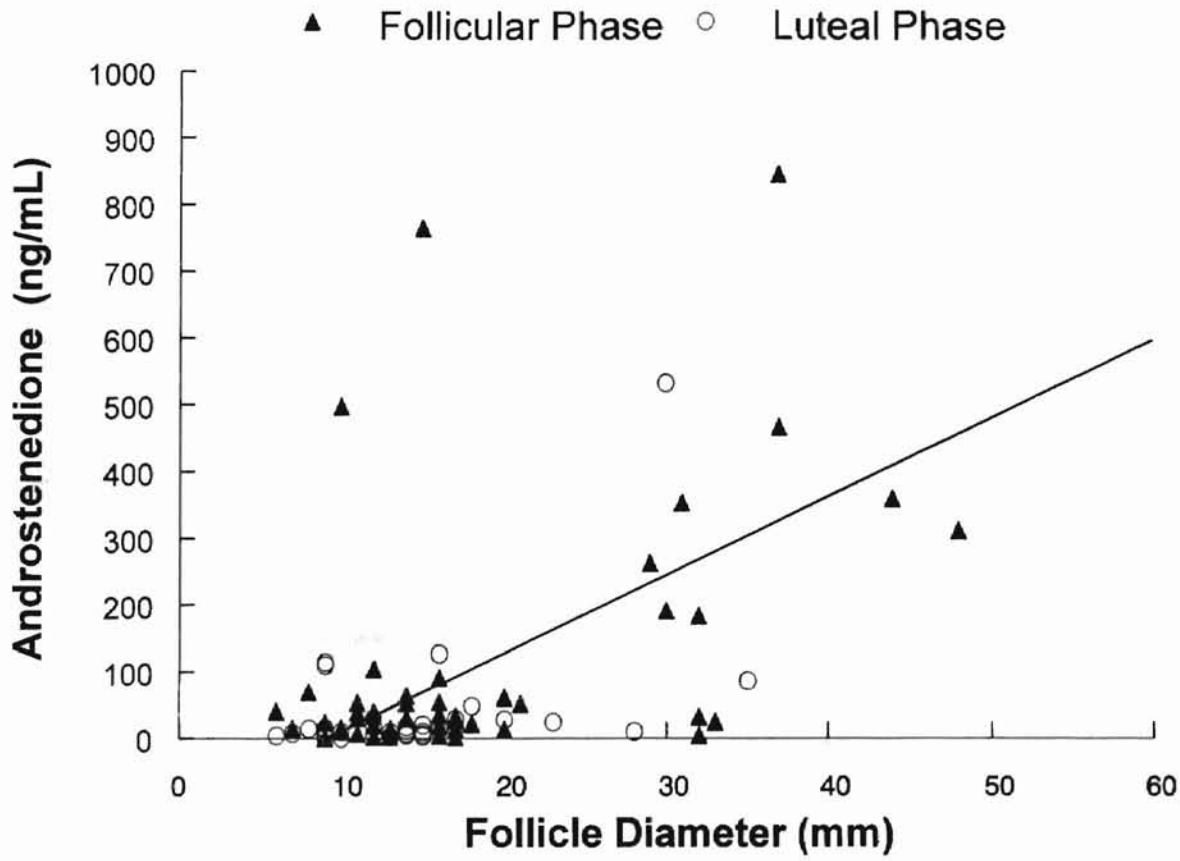

Figure 2. Linear regression analysis of androstenedione concentration in follicular fluid. Androstenedione concentration did not differ between follicular and luteal phases and is represented by the line y = 11.74x - 107.03.

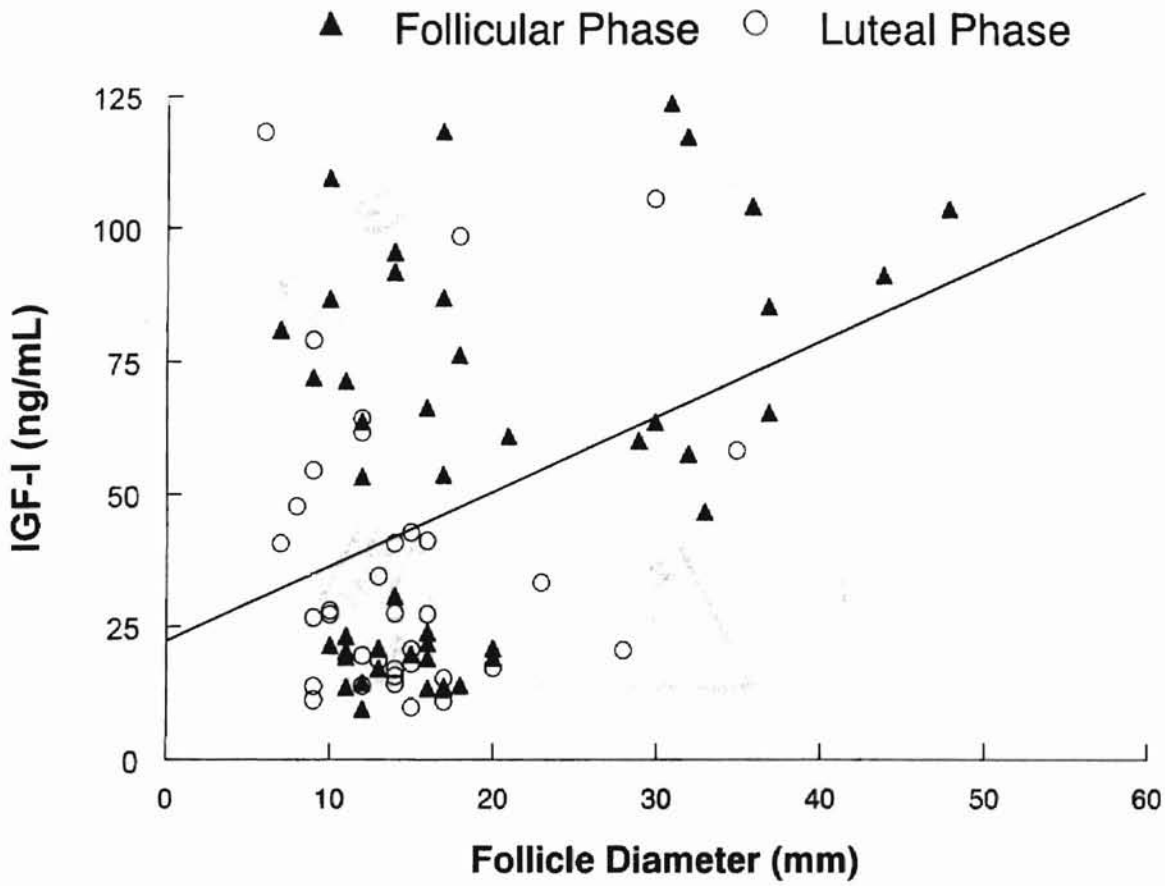

Figure 3. Linear regression analysis of IGF-I concentration in follicular fluid. IGF-I concentration did not differ between follicular and luteal phases and is represented by the line y = 1.41x – 22.32.

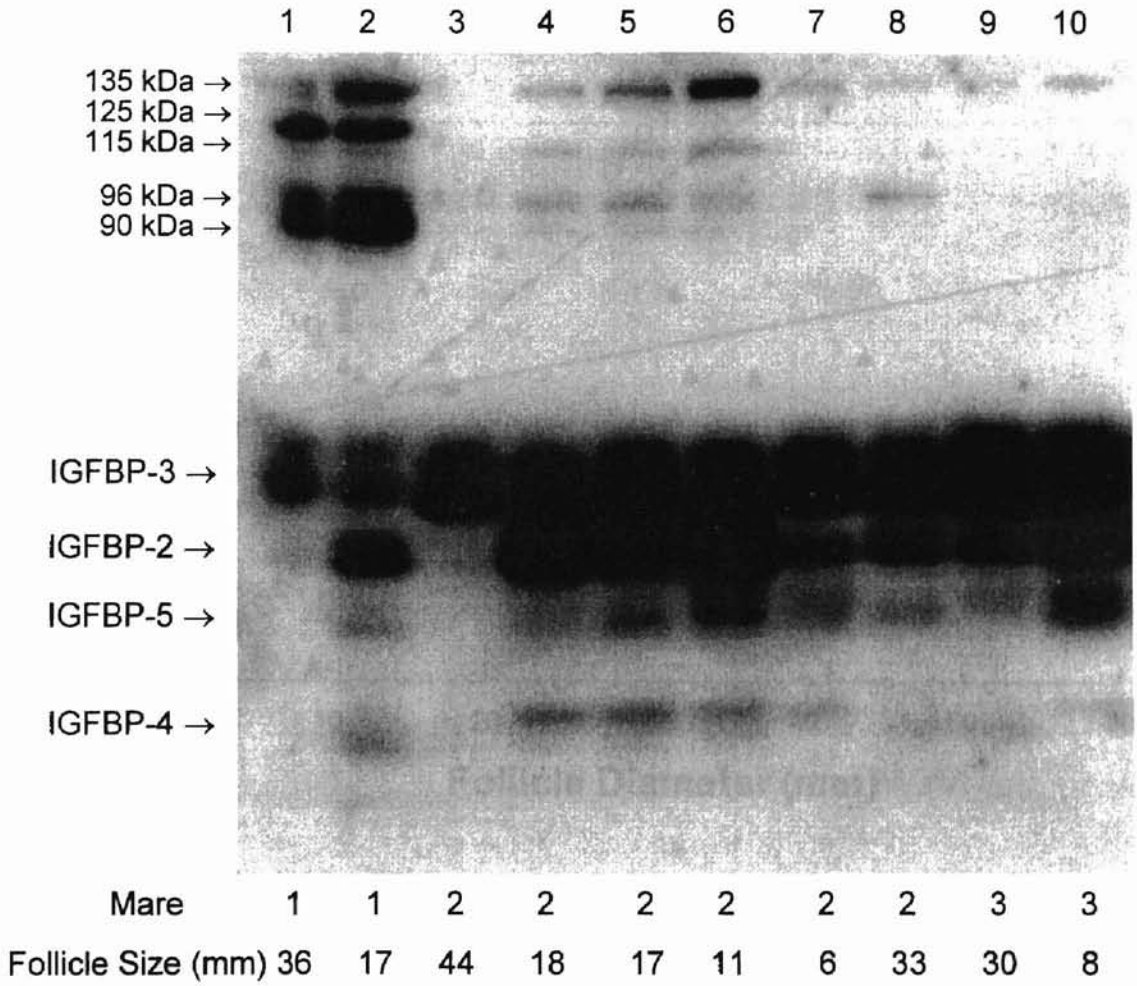

Figure 4. Representative ligand blot of IGFBPs present in equine follicular fluid.

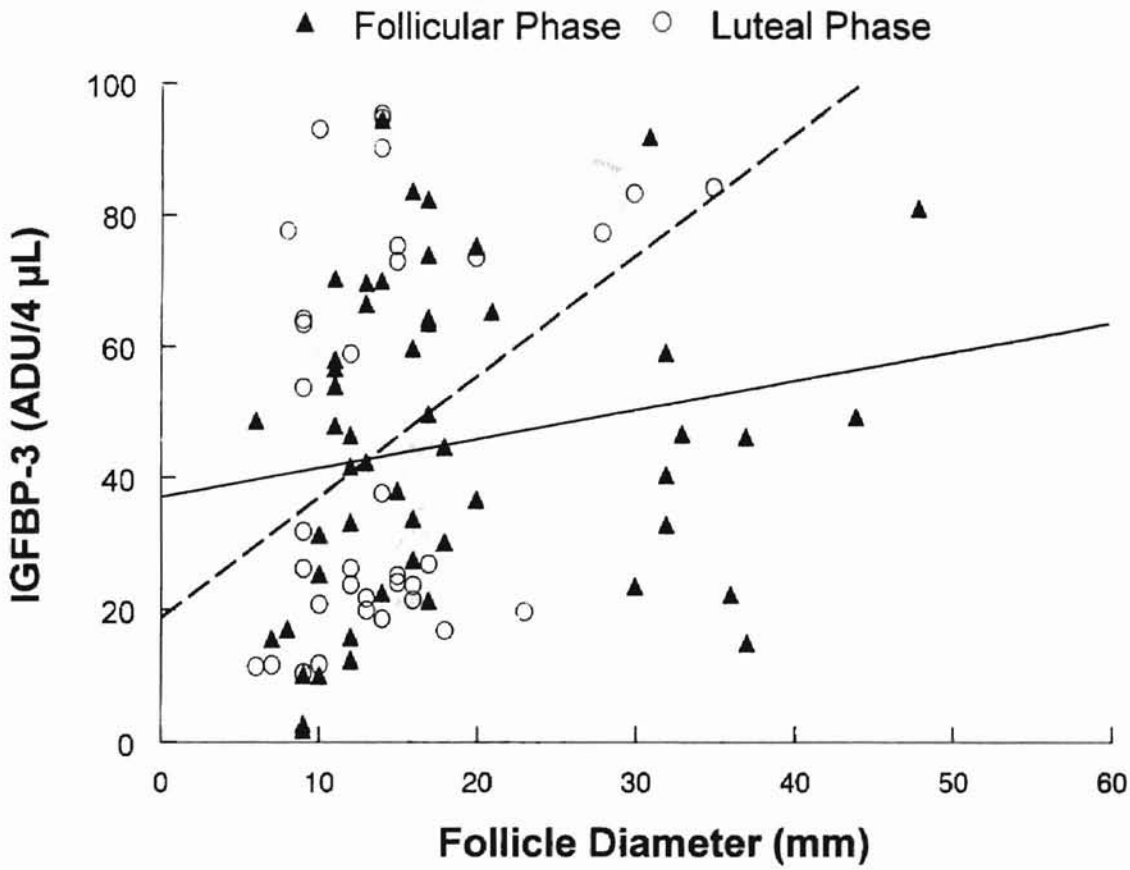

Figure 5. Linear regression analysis of IGFBP-3 concentration in follicular fluid. IGFBP-3 concentration in follicles during the follicular phase (——) is represented by the line y = 0.44x + 37.11. IGFBP-3 concentration in follicles during the luteal phase (– – –) is represented by the line y = 1.83x + 18.77.

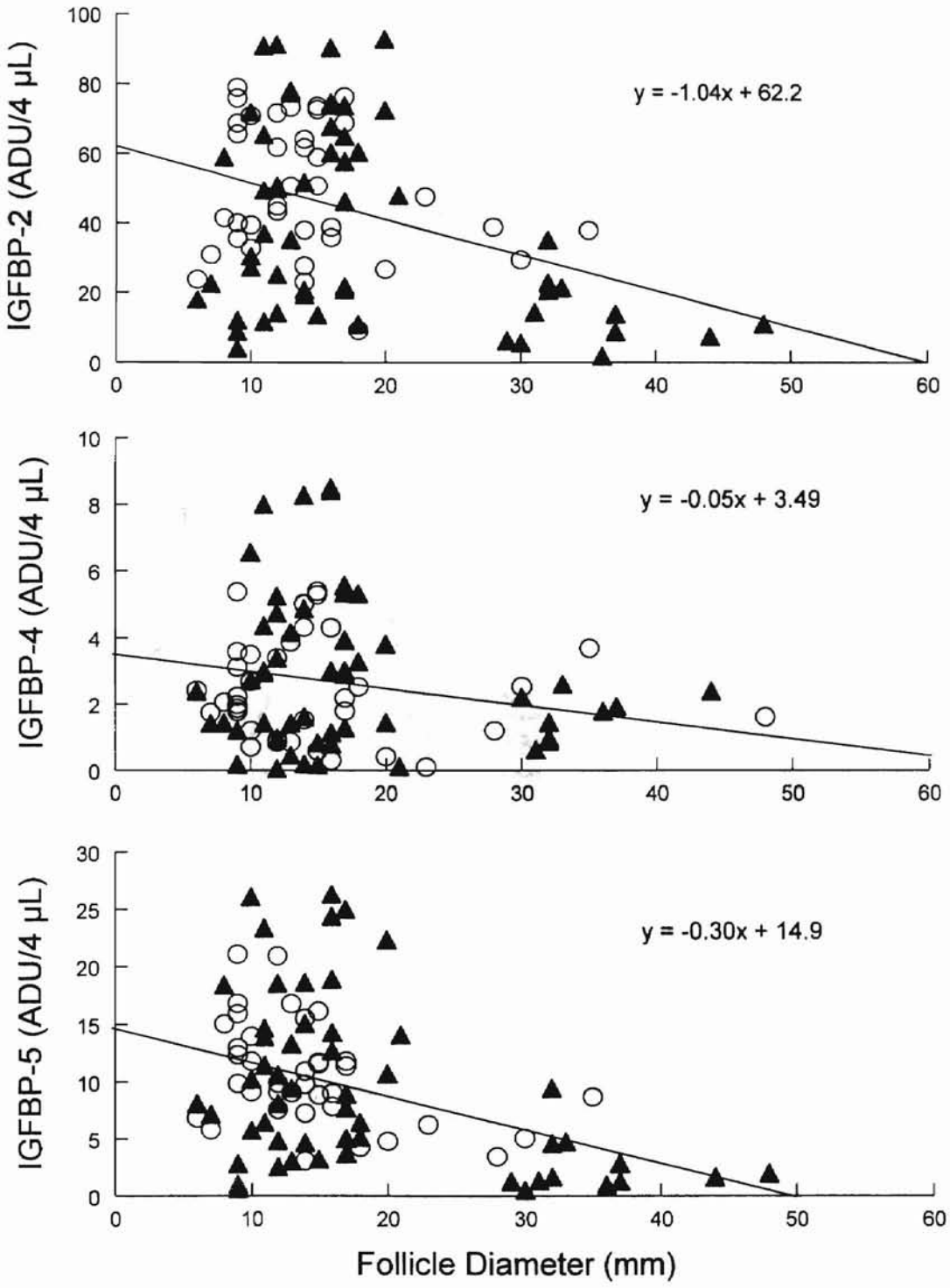

Figure 6. Linear regression analysis of IGFBP-2, -4 and –5 concentrations in equine follicular fluid. In each panel, the regression line represents IGFBP concentration during the combined follicular and luteal phases.

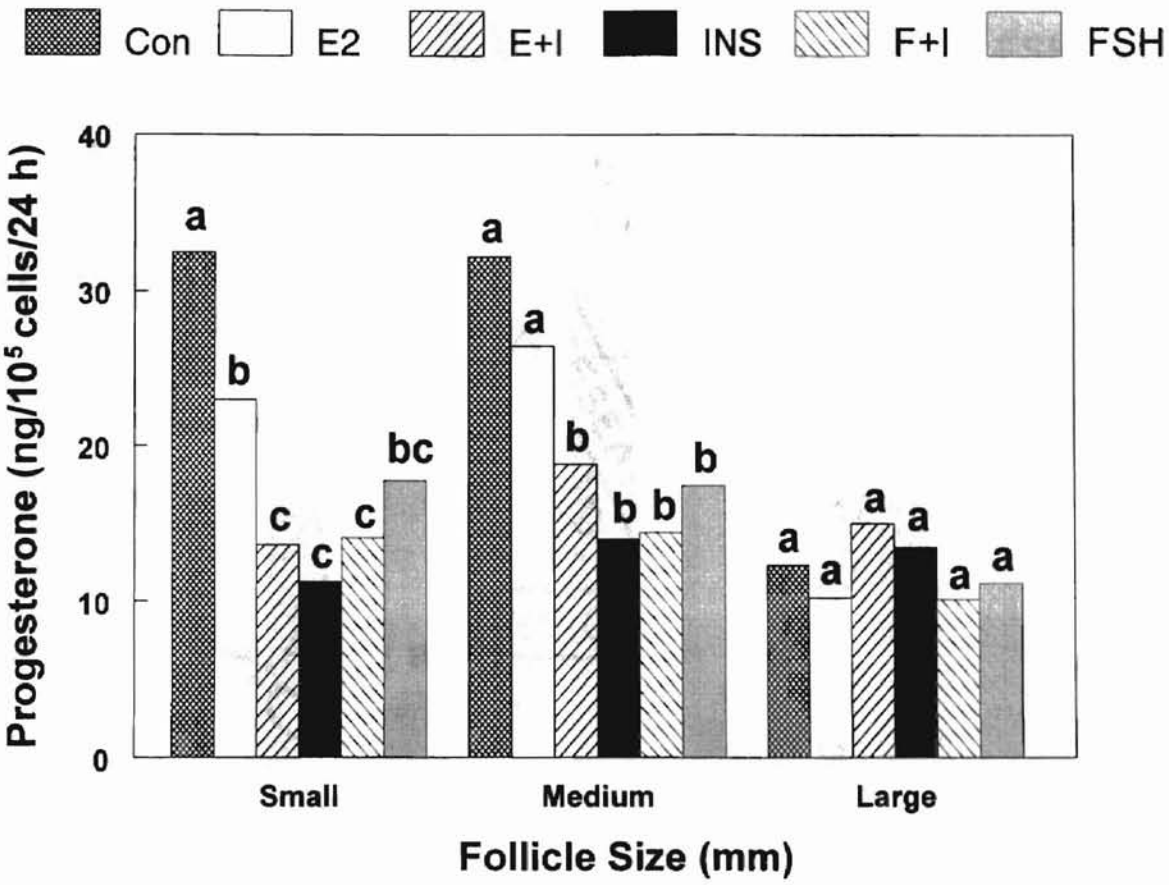

Figure 7. Effects of insulin (100 ng/mL), estradiol (500 ng/mL), and/or FSH (50 ng/mL) on progesterone production by granulosa cells collected from small (6 to15 mm), medium (16 to 25 mm), and large (25 to 48 mm) follicles of mares. Pooled SEM = 5.0 ng/10^5 cells/24 h.

[a, b, c] Within a follicle size, means without a common superscript differ ($p<0.05$).

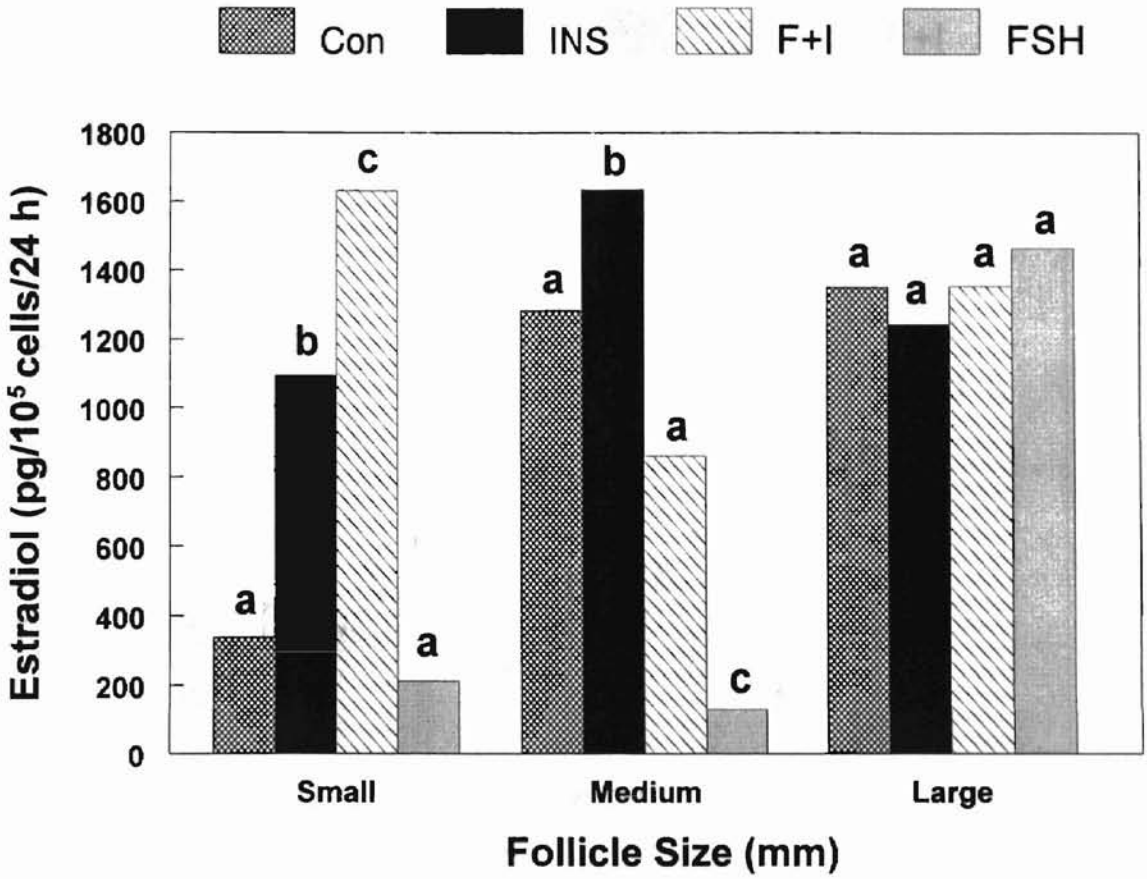

Figure 7. Effects of insulin (100 ng/mL) and/or FSH (50 ng/mL) on estradiol production by granulosa cells collected from small (6 to15 mm), medium (16 to 25 mm), and large (25 to 48 mm) follicles of mares.
Pooled SEM = 539 pg/10^5 cells/24 h.
[a, b, c] Within a follicle size, means without a common superscript differ ($p<0.05$).

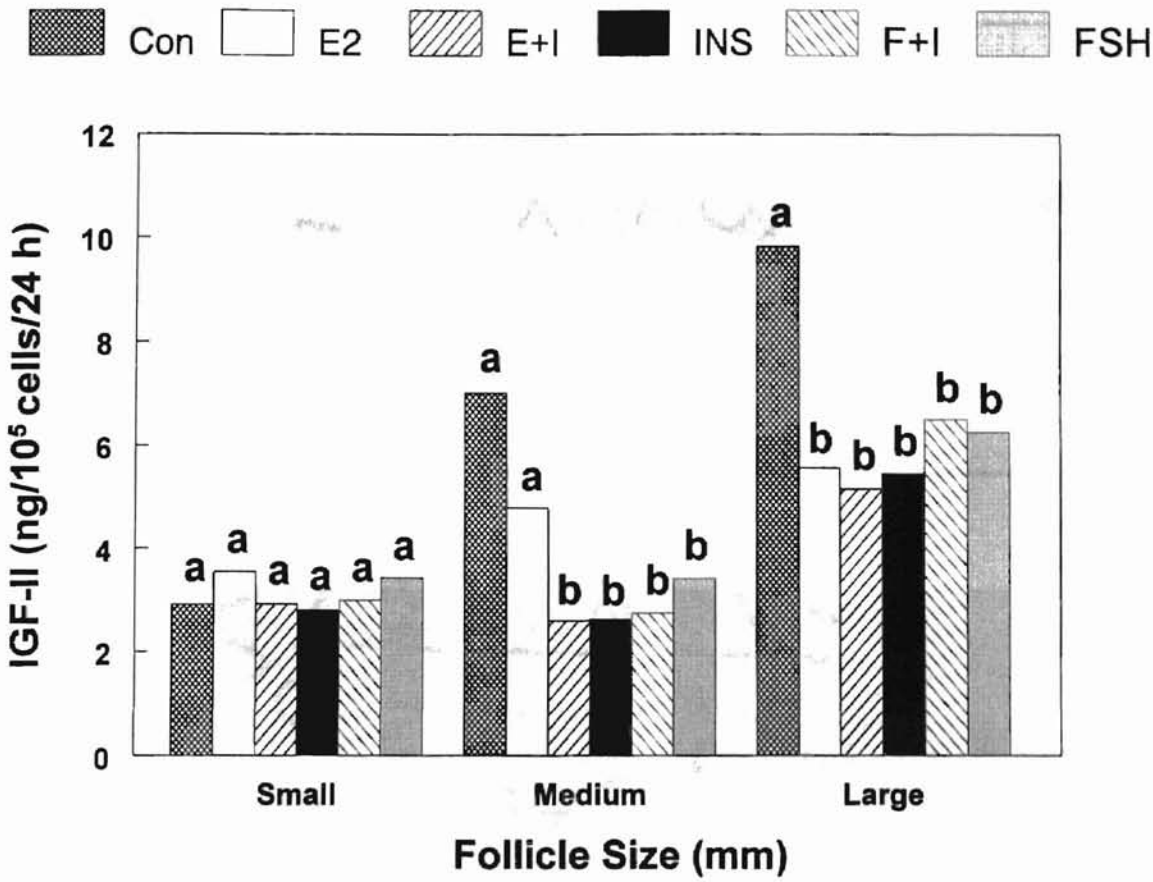

Figure 9. Effects of insulin (100 ng/mL), estradiol (500 ng/mL), and/or FSH (50 ng/mL) on IGF-II production by granulosa cells collected from small (6 to15 mm), medium (16 to 25 mm), and large (25 to 48 mm) follicles of mares. Pooled SEM = 1.2 ng/10^5 cells/24 h.

[a, b] Within a follicle size, means without a common superscript differ ($p<0.05$).

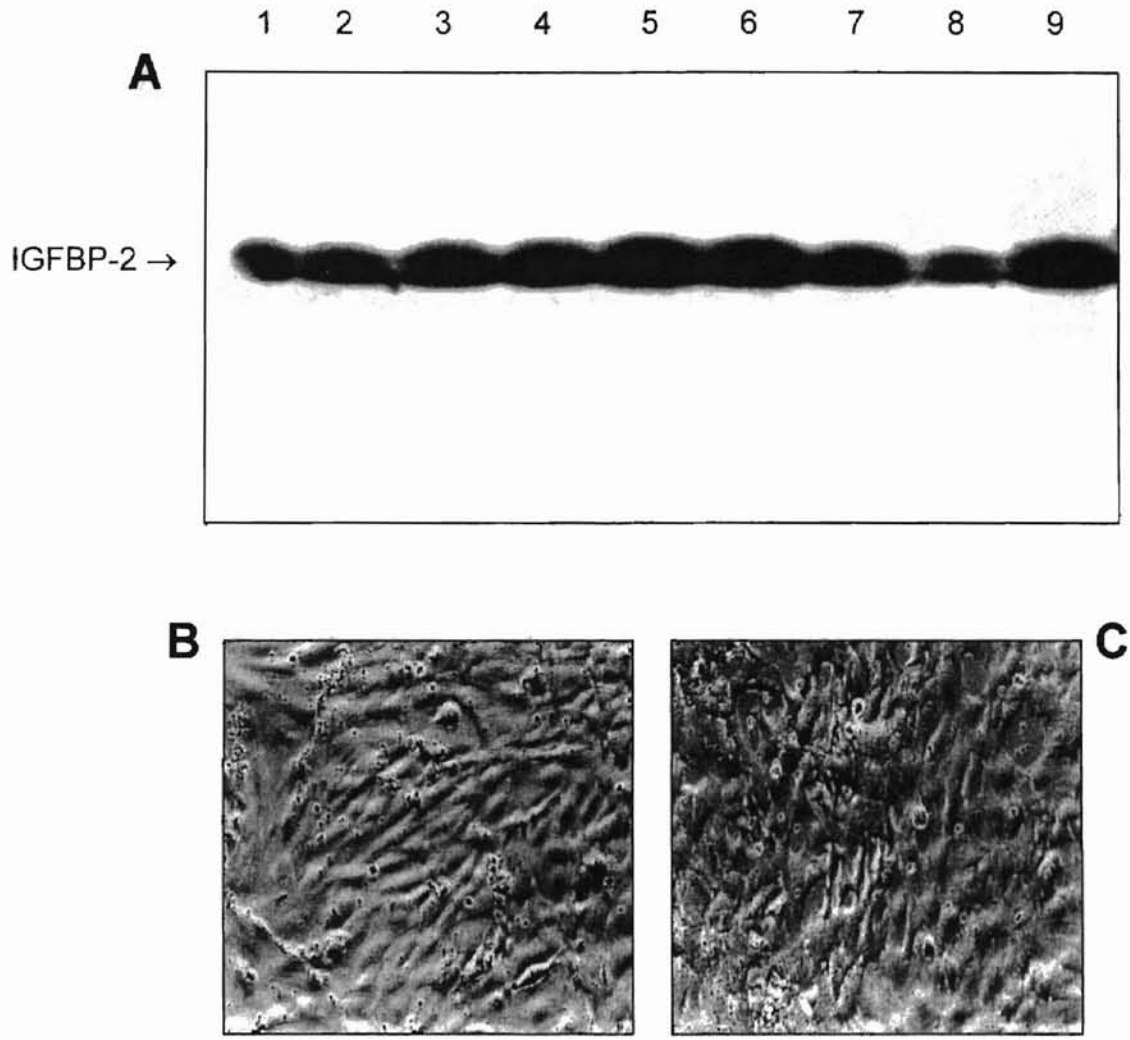

Figure 10. Panel A: representative ligand blot of IGFBP production by equine granulosa cells from small follicles. Treatments were as follows: lanes 1 and 2 = control, lanes 3 and 4 = estradiol, lanes 5 and 6 = insulin, lane 7 = estradiol+insulin, lane 8 = FSH, lane 9 = FSH+insulin. Panel B: representative example of control cultures of equine granulosa cells from small follicles under phase contrast microscopy (200x). Panel C: representative example of insulin treated cultures of equine granulosa cells from small follicles under phase contrast microscopy (200x).

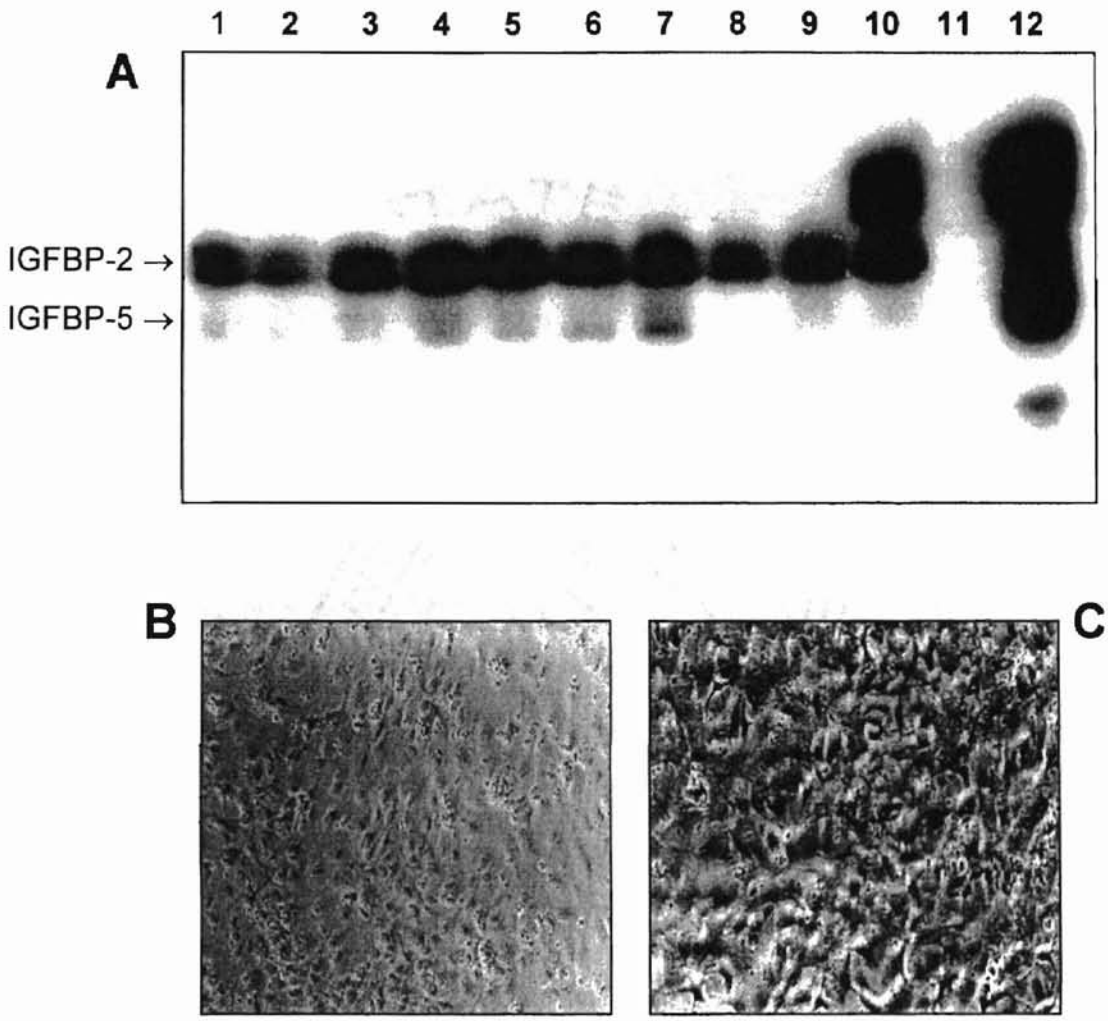

Figure 11. Panel A: representative ligand blot of IGFBP production by equine granulosa cells from medium follicles. Treatments were as follows: lane 1 = control, lane 2 = estradiol, lane 3 = insulin, lanes 4 and 5 = estradiol+insulin, lanes 6 and 7 = FSH, lanes 8 and 9 = FSH + insulin, lane 10 = equine follicular fluid, lane 11 = no sample, lane 12 = bovine follicular fluid. Panel B: representative example of control cultures of equine granulosa cells from medium follicles under phase contrast microscopy (200x). Panel C: representative example of insulin treated cultures of equine granulosa cells from medium follicles under phase contrast microscopy (200x).

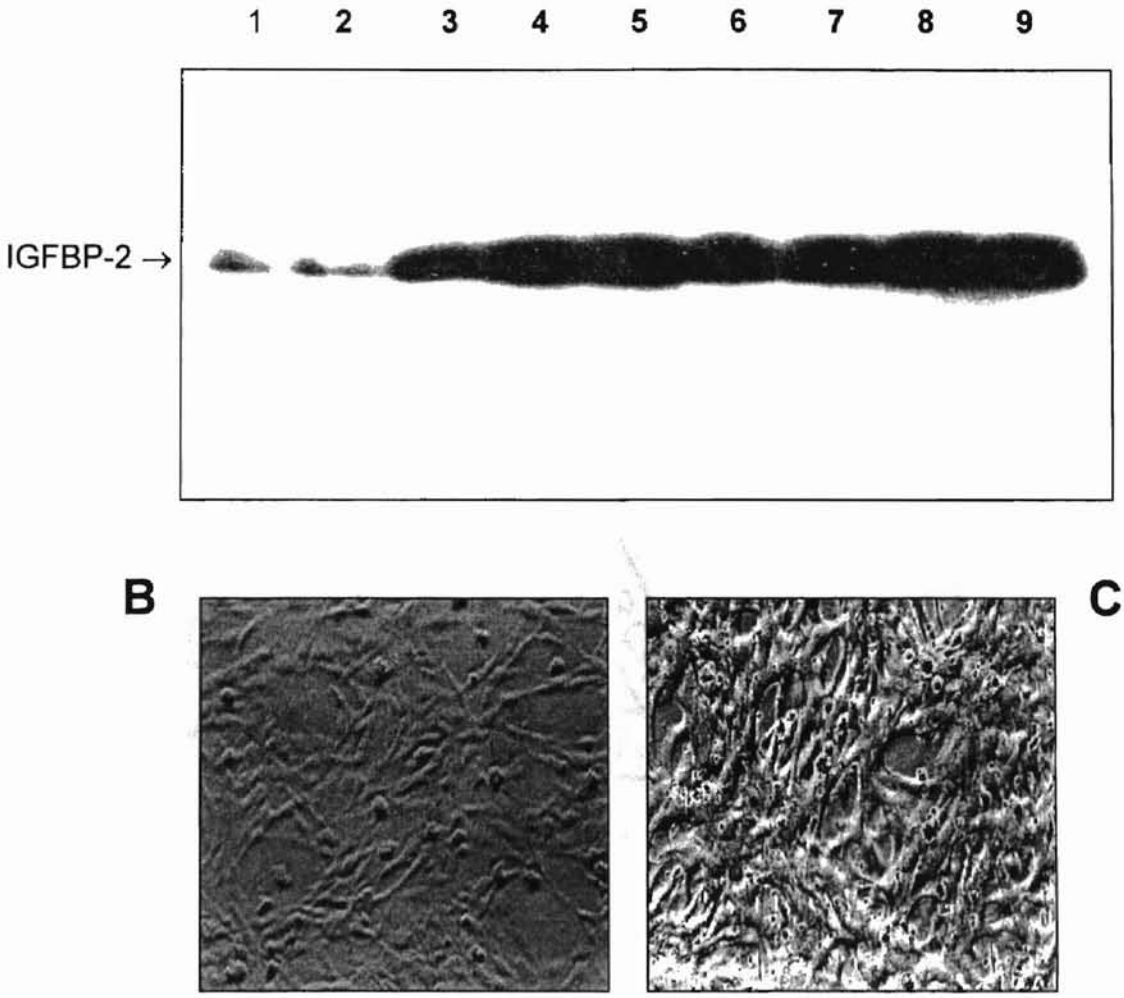

Figure 11. Panel A: representative ligand blot of IGFBP production by equine granulosa cells from large follicles. Treatments were as follows: lane 1 = control, lane 2 = estradiol, lane 3 = insulin, lanes 4 and 5 = estradiol+insulin, lanes 6 and 7 = FSH, lanes 8 and 9 = FSH+insulin. Panel B: representative example of control cultures of equine granulosa cells from large follicles under phase contrast microscopy (200x). Panel C: representative example of insulin treated cultures of equine granulosa cells from large follicles under phase contrast microscopy (200x).

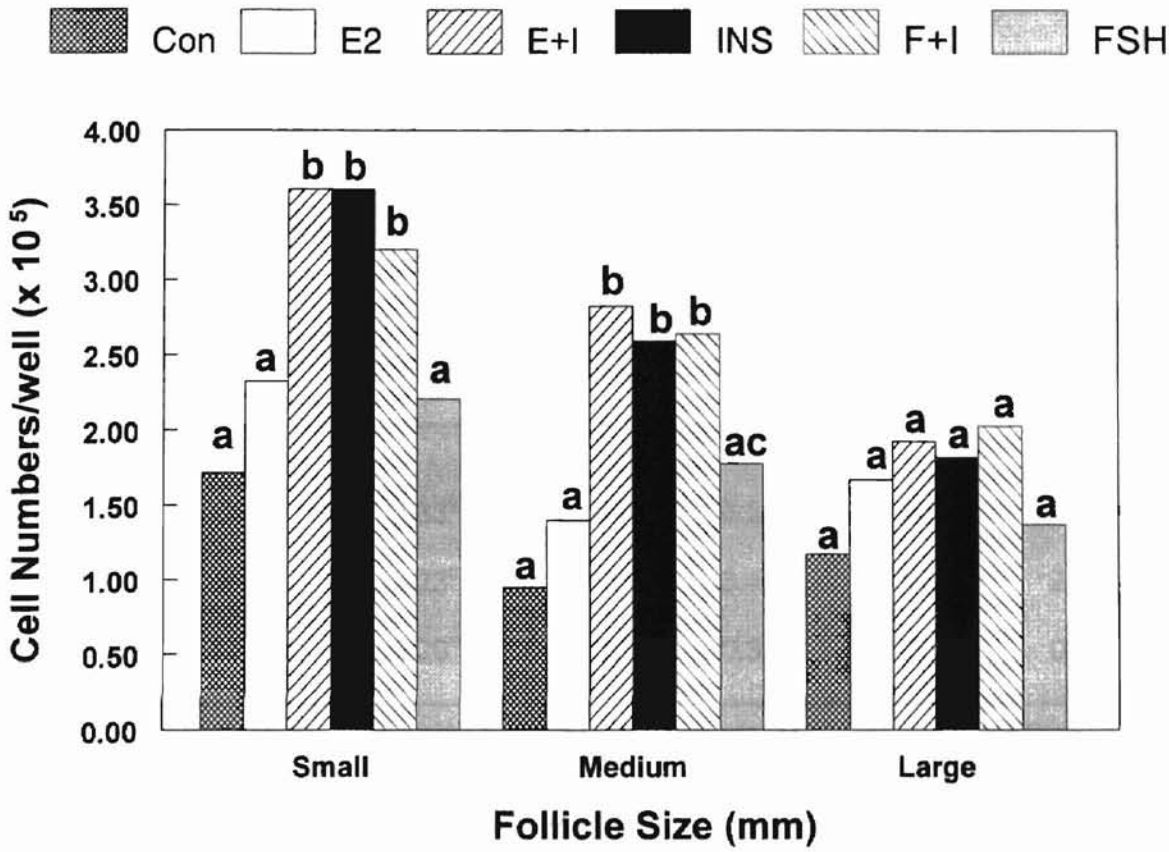

Figure 9. Effects of insulin (100 ng/mL), estradiol (500 ng/mL), and/or FSH (50 ng/mL) on granulosa cell numbers from small (6 to15 mm), medium (16-25 mm), and large (25 to 48 mm) follicles of mares. Pooled SEM = 1.2 ng/10^5 cells/24 h.

[a, b] Within a follicle size, means without a common superscript differ ($p<0.05$).

Table 1
Effect of estradiol, insulin and FSH on granulosa cell production of IGF-I, IGFBP-2 and IGFBP-5

	Treatments[1]					
	Control	Estradiol	Insulin	FSH	INS+E_2	INS+FSH
IGF-I[2]	0.39[a]	0.33[a]	0.25[b]	0.32[a]	0.25[b]	0.21[b]
IGFBP-2	2.50[c]	3.23[d]	2.46[c]	3.40[d]	2.85[c]	2.32[c]
IGFBP-5[4]	0.21[c]	0.20[c]	0.15[c]	0.32[d]	0.20[c]	0.21[c]

[1] Because follicle size was not significant, data were pooled across follicle sizes
[2] units = ng/10^5 cells/24 h; pooled SEM = 0.05.
[3] units = ADU/10^5 cells/24 h; pooled SEM = 0.43; ADU = arbitrary densitometric units.
[4] units = ADU/10^5 cells/24 h; pooled SEM = 0.10.
[a, b] Within a row, means without a common superscript differ ($p<0.05$).
[c, d] Within a row, means without a common superscript differ ($p<0.10$).

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Folliculogenesis is a complex process involving the interaction of a multitude of factors. During the estrous cycle of the mare, one follicle is selected from a cohort of follicles to become dominant, while the remaining cohort, or subordinate, follicles become atretic and regress. During preovulatory development in the mare, follicular fluid IGF-I levels increase whereas IGFBP levels decrease. In cattle, it has been hypothesized that estradiol and the gonadotropins induce changes in the amount of IGF-I and IGFBPs produced by granulosa and thecal cells during follicular devlopment. However, one question remains: what factors determine selection of the follicle?

The present study evaluated cultured equine granulosa cells to determine intrafollicular production and hormonal regulation of the insulin-like growth factor system and steroidogenesis in equine follicles in relation to follicle size and estrous cycle stage. IGF-I, IGF-II, IGFBP-2, -4 and –5 were present in equine follicle fluid and both stage of the estrous cycle and follicle diameter influenced concentrations of steroids and components of the IGF system. Furthermore, IGF-I, IGF-II, IGFBP-2 and –5 were produced by equine granulosa cells and steroidogenesis and(or) the IGF system of equine granulosa cells were

differentially regulated by FSH and estradiol. The conclusion to obtain from this work is that the insulin-like growth factor system and steroidogenesis are hormonally regulated in equine follicles. Decreased levels of low molecular weight IGFBPs in large estrogen-active follicles may allow an increase in free IGF-I and thus increase gonadotropin responsiveness and growth of the future dominant follicle. In contrast, increased levels of the low molecular weight IGFBPs in small cohort follicles may act to sequester IGF-I causing a low responsiveness to IGF-I and FSH, thus slowing growth rate of future subordinate follicles.

VITA

Tonya R. Davidson

Candidate for the Degree of

Master of Science

Thesis: EFFECT OF FOLLICLE SIZE ON IN VITRO PRODUCTION OF STEROIDS AND INSULIN-LIKE GROWTH FACTOR (IGF)-I, IGF-II AND THE IGF BINDING PROTEINS (IGFBPs) BY EQUINE OVARIAN GRANULOSA CELLS

Major Field: Animal Science

Biographical:

Personal Data: Born in Lufkin, Texas on September 24, 1972, the daughter of James and Linda Davidson.

Education: Graduated from Southside High School, Fort Smith, Arkansas in May, 1991; received an Associate of Science degree from Carl Albert State College, Poteau, Oklahoma in May, 1993; received a Bachelor of Science degree in Animal Science from Oklahoma State University, Stillwater, Oklahoma in December, 1997. Completed the requirements for the Master of Science degree with a major in Animal Science at Oklahoma State University in December 2000.

Professional Memberships: Society for the Study of Reproduction, OSU Graduate Student Association