Effect of progesterone priming on ovarian angiogenic factors during late follicular and early luteal development

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CHAPTER 1

Introduction and Literature Review

1.1 General Introduction

Infertility is a major issue in both human and animal medicine with a great economic impact on reproduction. In order to better understand the common causes of infertility it is necessary to understand the basic physiology underlying the complex process of folliculogenesis and luteogenesis (Campbell et al., 2003).

The lack of extensive understanding of the factors involved in regulating follicular and oocyte maturation has resulted in the slow process of developing *in vitro* systems for the study of folliculogenesis (Campbell et al., 2004). However, domestic ruminants represent a valuable *in vivo* model system for the study of the endocrine and local mechanisms regulating follicular development not only in ruminants but also in humans (Campbell et al., 2003) and have thus been extensively used in reproduction research.

Improved understanding of the basic cellular mechanisms of ovarian physiology may improve biotechnology strategies in livestock reproduction and enhance fertility protocols for endangered species. Similarly, a better understanding of these reproductive processes could lead to innovative strategies to improve reproductive health in women (McLaughlin and McIver, 2009, Stellflug et al., 1997, Campbell et al., 2003, Skinner, 2005).

1.2 Endocrine control of the oestrous cycle in sheep

1.2.1 Seasonality of the breeding season

Seasonal reproduction is most evident in wild species as domestication and artificial selection have, over the centuries, contributed to minimize the effects of season on reproductive activity. Seasonal breeders rely on environmental cues to synchronize their fertile period. Photoperiod is the most common synchronizing agent and entrains the endogenous rhythm of reproductive activity as it shows minimum variation through the years as opposed to temperature and food availability (Rosa and Bryant, 2003). Species that rely on photoperiod to synchronize their breeding activity are categorized into long or short day breeders. Deer, goats and

sheep are considered short-day breeders and become sexually active in response to decreasing day length in late summer to early autumn (Rosa and Bryant, 2003, Thiery et al., 1995, Stellflug et al., 1997).

Sheep breeds originating from temperate climate in mid or high latitudes (greater than 35°) are seasonal breeders. On the other hand, in tropical and sub-tropical environments (between 35° North and 35° South) ewes are either completely aseasonal or intermittently polyoestrus. While the breeding season of these animals can still be initiated by declining day length, food quality and availability play a much more important role in regulating breeding activity than photoperiod. In general, the higher the latitude the greater the photodependence and the more restricted the period of breeding activity. British sheep breeds demonstrate a distinct seasonality with long anoestrous periods, while Mediterranean breeds are comparatively less seasonal (Rosa and Bryant, 2003).

Photic stimuli (light) is received by the retinal photoreceptors and transmitted to the suprachiasmatic nuclei of the hypothalamus which regulates the endogenous circadian rhythm. The message is then transmitted to the pineal gland which converts the neural information into a hormonal signal in the form of melatonin. Melatonin is secreted following a circadian rhythm with significant secretion occurring only in the absence of daylight (Rosa and Bryant, 2003, Stellflug et al., 1997, Thiery et al., 1995). Melatonin influences the onset of the breeding season by decreasing the sensitivity of the hypothalamus to oestrogen negative feedback and increasing the pulse frequency of hypothalamic gonadotrophin releasing hormone (GnRH), re-establishing regular cyclicity (Stellflug et al., 1997, Thiery et al., 1995, Moenter et al., 1992, Rosa and Bryant, 2003).

1.2.2 Hypothalamic-Adenohypophyseal-Ovarian axis

1.2.2.1 Hypothalamus

Gonadotrophin releasing hormone (GnRH), a decapeptide produced and released by the hypothalamus, is responsible for the majority of the endocrine events that occur during the oestrous cycle. Increases in GnRH pulses result in an increase in the

hypophyseal gonadotrophin release and consequent follicular development and ovulation. A variation in the frequency and amplitude of GnRH pulses alters gonadotrophin secretion by the hypophysis (Thiery et al., 1995, Stellflug et al., 1997, Caldani et al., 1995, Caraty et al., 1995, Stabenfeldt and Edquist, 1996, Tilbrook and Clarke, 1995). As follicular development progresses and oestrogen secretion by the dominant follicle increases, there is a reduction in GnRH pulse amplitude and an increase in its frequency before the GnRH surge that causes the onset of the pre-ovulatory luteinizing hormone (LH) surge (Caraty et al., 1995).

1.2.2.2 Adenohypophysis

The gonadotrophins, produced by the adenohypophysis, are the follicular stimulating hormone (FSH) and the luteinizing hormone (LH). FSH is responsible for the earlier stages of growth and development of follicles while LH is mainly responsible for the final maturation of the follicle and causes the rupture of follicular wall and ovulation with consequent formation of a *corpus luteum* (CL). An important synergism exists between the two gonadotrophins. Follicles must be properly developed by FSH before LH induced ovulation can occur. An increase in oestrogen secretion and GnRH pulse frequency leads to the ovulatory LH surge. At this time the constant exposure of the hypophysis to elevated GnRH causes a decrease in its responsiveness to the decapeptide resulting in the termination of the pre-ovulatory LH surge (Moenter et al., 1992).

1.2.2.3 Ovary

During follicular development, the theca layer produces androgens which are converted by the granulosa cells of the growing follicle into oestrogen (17β-estradiol) (Driancourt, 2001). There is a significant seasonal variation in the responsiveness of the hypothalamic-hypophysis axis to the negative feedback action of oestrogen on LH secretion in sheep, which appears to be mainly dictated by the photoperiod (Webster and Haresign, 1983, Hansel and McEntee, 1977, Herbison, 1995). There are three components of oestrogen feedback on GnRH and gonadotrophin secretion:

(1) short-term negative feedback, (2) long-term negative feedback and (3) transient positive feedback. The positive feedback occurs in response to the rising levels of oestrogen during the follicular phase of the oestrous cycle while the low levels of oestrogen observed during the luteal phase and anoestrus result in the short and long-term suppression of GnRH and gonadotrophin release (Clarke et al., 2009). This negative feedback control on tonic LH secretion results from the combined action of oestrogen and progesterone during the breeding season, however during seasonal anoestrus, a marked change of hypothalamic responsiveness to oestradiol alone can be observed (Webster and Haresign, 1983). Oestrogen is also responsible for oestrous behaviour in all species including sheep, however, small amounts of progesterone priming are necessary for the induction of oestrus in sheep during the first reproductive cycle of the breeding season (Hansel and McEntee, 1977, Herbison, 1995, Stellflug et al., 1997, Legan et al., 1985).

The main developing follicle is also responsible for the production of inhibin. Inhibin is a glycoprotein hormone which inhibits the production and secretion by the pituitary gland of the gonadotrophins, especially FSH. Inhibin is a major selective feedback regulator of the secretion of FSH and its actions occur at the levels of the pituitary gland and not on the synthesis or secretion of GnRH (Stabenfeldt and Edquist, 1996).

An illustrative diagram of the hormonal interactions that occur during the breeding season can be seen in Figure 1.

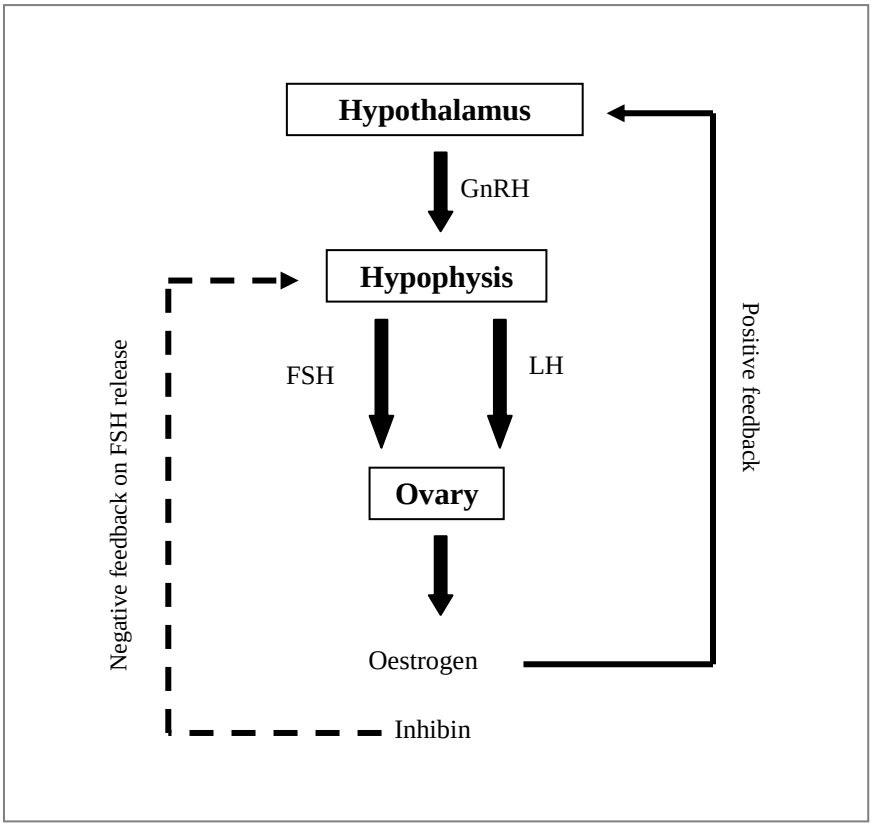


Figure 1. A diagrammatic representation of the hormonal interaction taking place within the hypothalamic-hypophyseal-ovarian axis of the ewe during the breeding season

1.2.3 Hormone interaction during anoestrus

During seasonal anoestrus, LH continues to be released but with lower frequency than during the follicular phase of the cycle during the breeding season (Rosa and Bryant, 2003). Progesterone levels remain virtually undetectable while FSH concentrations do not seem to vary from those found during the breeding season (Rosa and Bryant, 2003). Although progesterone levels are low and no gonadotrophin surge occurs, neither the ovary nor the hypothalamus are inactive (Rosa and Bryant, 2003). Follicular development does not stop, follicular growth and regression are taking place and mature follicles are present. These follicles are responsive to gonadotrophins and capable of ovulating if induced by GnRH or LH (Rosa and Bryant, 2003). The LH pulse system, although active, is compromised as GnRH and LH pulse frequencies are low. The mature follicles present during

anoestrus are capable of producing oestrogen which now has greater ability to suppress LH release. Therefore, growth of the dominant follicle can not be maintained for a long enough period to increase oestrogen concentrations and thus exert positive feed-back on the hypothalamus-hypophysis axis and induce a pre-ovulatory LH surge (Rosa and Bryant, 2003). The negative feedback of oestrogen on LH secretion is brought about via a change in the pattern of melatonin secretion which is associated with a reduction in GnRH pulse secretion rather than a reduction of pituitary response to GnRH. As sheep GnRH neurons do not express steroid hormone receptors, it has been suggested that the response to oestrogen feedback that leads to anoestrus involves afferents to GnRH neurons which have the potential to receive and transmit steroidal feedback signals (Rosa and Bryant, 2003). Kisspeptin cells posses oestrogen receptors and provide direct synaptic output to GnRH neurons stimulating GnRH release. Furthermore, positive feedback of oestrogen on GnRH release is associated with increased kisspeptin gene (*Kiss1*) expression while the negative feedback of oestrogen is associated with reduced *Kiss1* and kisspeptin levels (Clarke et al., 2009).

At the end of anoestrus, with a change in the pattern of melatonin secretion, responsiveness to oestrogen negative feedback diminishes increasing GnRH pulse secretion and therefore LH pulses. As follicles undergo further development in the presence of increased LH, oestrogen production is enhanced leading to the induction of the first pre-ovulatory LH surge and re-establishment of cyclicity (Rosa and Bryant, 2003). Absence of ovulation during seasonal anoestrus is thought to be due to reduced frequency of LH pulses (Wallace et al., 1986, McNatty et al., 1984, McLeod et al., 1982a).

The transition from anoestrus to reproductive activity is gradual with the occurrence of an initial short cycle as the first CL regresses prematurely 5-6 days after its formation. It is only after the 1st ovarian cycle that behavioural oestrus is exhibited (Rosa and Bryant, 2003).

1.3 Follicular growth and development

Although differences exist between ruminant species, general follicular dynamics are very similar. Follicles grow in a wave-like fashion with periodic FSH surges being associated with follicular wave development. Dominant follicle selection involves a decline in FSH and acquisition of LH responsiveness. Periodic anovulatory follicular waves continue to emerge until the occurrence of a LH surge during the follicular phase of the oestrous cycle which leads to ovulation (Adams, 1999).

1.3.1 Stages of follicular development

Folliculogenesis begins with the recruitment of pre-granulosa cells to form the primordial follicles in the foetus and neonatal ovary of mammals (McLaughlin and McIver, 2009). Follicular development involves a gonadotrophin independent (primordial follicles) and a gonadotrophin dependent (primary, secondary and tertiary follicles) phase before the follicle is ready for ovulation (Figure 2).

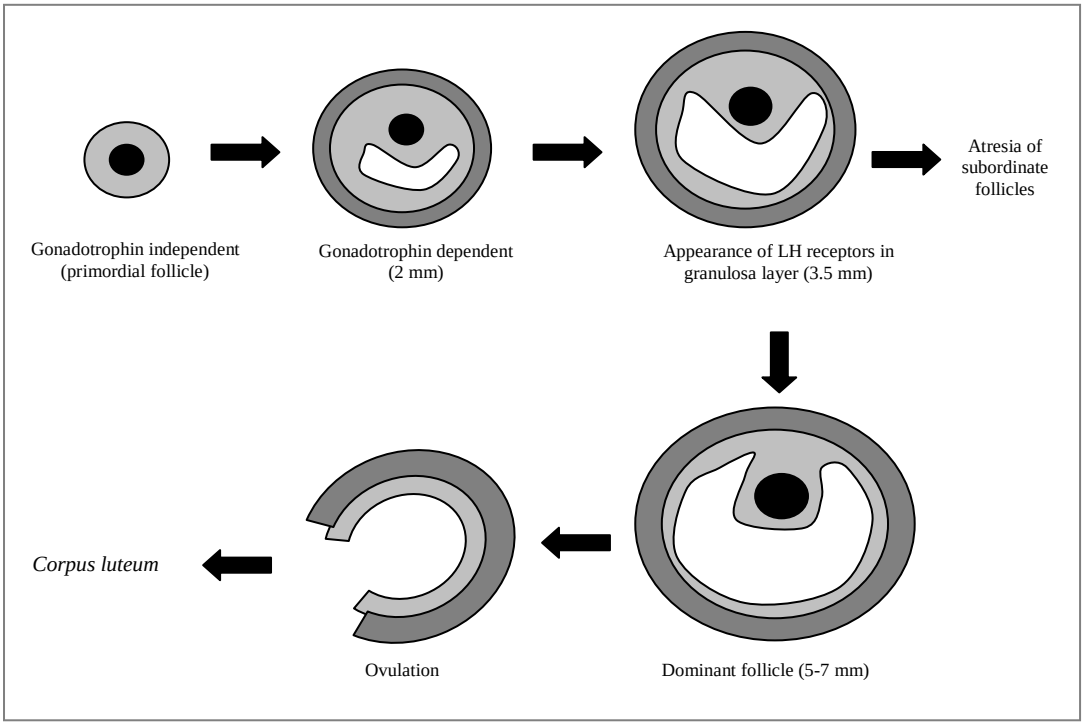


Figure 2. Diagram illustrating follicular development and *corpus luteum* formation in the sheep during the breeding season.

1.3.1.1 Gonadotrophin independent phase

During the gonadotrophin independent phase of follicular development the oocyte increases in size and activity and the follicular cells which initially formed around the oocyte begin to grow and divide, transforming into granulosa cells (Stabenfeldt and Edquist, 1996). The first follicles form when primary oocytes are enveloped by a single layer of flattened granulosa cells and are denominated primordial follicles (Fortune, 2003, Stevens and Lowe, 1993)

The primary determinant of reproductive performance in female mammals is the size and longevity of the primordial follicle pool (McLaughlin and McIver, 2009). It has been widely accepted that an animal is born with a finite primordial follicle population. However, some data have been generated that support the belief that post-natal oogenesis can occur in mice (McLaughlin and McIver, 2009). So far it has not been proved that these newly formed follicles can contribute to the pool of ovulated oocytes but it is thought that they may play a role in providing support for the depleted ovarian reserve (McLaughlin and McIver, 2009, Lee et al., 2007).

It has been reported that in humans and most domestic animals the first primordial follicles can be detected as early as mid-gestation but only after 2 to 4 days post-birth in rodents, and that specific factors expressed by the oocyte regulate primordial follicle formation (McLaughlin and McIver, 2009, Fortune, 2003).

Up to now, many aspects of primordial follicle population dynamics remain unanswered. From the pool of primordial follicles packed in the ovarian cortex some grow to develop into primary, secondary and antral follicles whilst others remain quiescent (Stevens and Lowe, 1993). The "production line" hypothesis suggests that the first oocytes to enter meiotic arrest during embryonic gonad development are the first oocytes to activate in adult life. This hypothesis is based on the belief that oocytes produced in late mammalian life are more likely to be chromosomally imbalanced and thus dysfunctional. This hypothesis is supported by studies that found that oocytes close to the corticomedulary junction are amongst the first to activate whereas oocytes located further within the cortex activate later in life. This hypothesis that follicles do not activate and mature randomly has been contested by *in vitro* studies that demonstrate that, in culture, the majority of primordial follicles

activate simultaneously and thus it is suggested that initiation of primordial follicle growth was subject to extreme factors (McLaughlin and McIver, 2009). However, once activated to grow the oocyte controls the rate of follicular development. Furthermore, primordial follicle activation also appears to require close communication with somatic cells and its factors (McLaughlin and McIver, 2009).

The assembly of primordial follicles early in ovarian development and the transition from primordial to primary follicle are critical processes in ovarian biology. Primordial follicle assembly and development are coordinated by locally produced paracrine and autocrine growth factors. Progesterone is known to influence follicular assembly (Skinner, 2005); however, the re-establishment of growth and development of primordial follicles is not fully understood. A complex network of cell to cell interactions is required to control the primordial to primary follicle transition (Skinner, 2005, Stabenfeldt and Edquist, 1996).

It has been suggested that a gonadotrophin-independent intraovarian feedback loop exists regulating both the rate of primordial follicle recruitment and early follicular development. However, the factors involved in this feedback are still unknown (Campbell et al., 2004).

1.3.1.2 Gonadotrophin dependent phase

When the primordial follicles leave the resting pool in the ovarian cortex the flattened granulosa layer becomes cuboidal and starts to show signs of proliferation (Fortune, 2003, Stevens and Lowe, 1993). These follicles are then considered primary follicles.

These primary follicles further develop into secondary follicles which start with the formation of a second layer of granulosa cells, progress through the addition of six or seven layers and terminate with the gradual development of an antral cavity (Fortune, 2003). As the follicle matures and the granulosa layer increases in thickness, the ovarian stromal cells that surround the follicle develop into 2 layers. The inner layer of stromal cells is denominated theca interna and is the source of androgens which will be converted into oestrogen by aromatase, produced by the granulosa cells. The theca externa corresponds to the outer stromal layer and has no

steroidal secretory function (Stevens and Lowe, 1993). While in mice a distinct theca layer can be observed during the late primary/early secondary stage of follicular development, primate, bovine and ovine follicles only appear to develop distinct thecal layers during the mid or late secondary stage (Fortune, 2003).

In secondary follicles the oocyte is surrounded by proliferating granulosa cells (McLaughlin and McIver, 2009), the theca layer has its own vascular network but the granulosa layer remains avascular (Kaczmareck et al., 2005)

The follicular stages can be classified according to many different methods developed in many different species. Based on histological findings Braw-Tal and Yossefi (1997) have proposed the classification of the different bovine follicular stages into primordial, primary, small pre-antral, large pre-antral and small antral as detailed in Table 1. This classification has also been used as a framework to describe ovine follicular development (Fortune, 2003).

Table 1. Classification and characterization of small bovine follicles according to Braw-Tal Yossefi (1997)

Follicle	Layers of granulosa cells	Follicle diameter (μm)	Defined theca interna
Primordial	1	< 40	-
Primary	1-1.5	40-80	-
Small pre-antral	2-3	81-130	-
Large pre-antral	4-6	131-250	+
Small antral	> 6	250-500	++

Under the appropriate gonadotropic stimulation, granulosa and theca cells of follicles continue to proliferate and differentiate until ovulation. Follicular angiogenesis is initiated early during follicular development and continues throughout follicle growth (Kaczmareck et al., 2005).

As the granulosa develops under the influence of FSH and oestrogen it begins to synthesize and release secretions that cause cell separation, resulting in the formation of the antrum (Stabenfeldt and Edquist, 1996). Once the antrum is formed, the follicles are denominated tertiary or Graafian follicles. The oocyte is separated from

the follicular fluid by layers of granulosa cells called *cumulus oophorus* and the large follicle, at this stage, is ready to ovulate (Stevens and Lowe, 1993).

In sheep and cattle the life-span for a primordial follicle to reach ovulation is approximately 4 to 6 months (Hunter et al., 2004, Campbell et al., 2004).

1.3.2 Hormonal regulation of folliculogenesis

FSH plays an important part in early follicular development (Fortune, 2003). However, studies have shown that early folliculogenesis is not FSH dependent (Campbell et al., 2000). Follicles are able to reach the small antral stage of development in the absence of gonadotrophins but follicles exposed to FSH appear healthier and have a larger number of proliferating cells than those deprived of the hormone (Campbell et al., 2000). Although not essential, FSH seems to work as a modulator of early folliculogenesis by influencing the size of the pre-antral population (McLaughlin and McIver, 2009) and the rate at which pre-antral follicle development proceeds (Campbell et al., 2000).

Follicular development prior to ovulation involves important morphological and functional changes in the theca and granulosa layers (Monget and Monniaux, 1995) and can be classified into 3 stages: recruitment, selection and dominance (Driancourt, 2001).

During the recruitment phase a pool of follicles is produced from which one or more pre-ovulatory follicles are eventually selected. Once selected, these follicles become dominant and progress towards ovulation while all other recruited follicles undergo atresia and regress. The process of recruitment and selection is controlled by endocrine and paracrine factors, including various intraovarian growth factors. In nature the number of ovulatory follicles produced per oestrous cycle is closely related to the species and breed of the animal. However, ovulation rate can be manipulated by administration of exogenous hormones (Hunter et al., 2004).

The follicular recruitment process is characterized by gonadotrophin-dependent folliculogenesis in which FSH is the key hormone involved. Follicles of 0.8 to 2 mm in diameter are sensitive to gonadotrophins but only become gonadotrophin dependent when they reach 2 mm in diameter in sheep and 3 to 4 mm in diameter in

cows (Driancourt, 2001, Hunter et al., 2004). FSH enables inhibin production and stimulates aromatase activity within the granulosa cells of the developing follicles. Androgens, produced by the theca layer under the influence of LH, are converted by aromatase into oestrogen. Oestrogen stimulates granulosa cell multiplication and additional FSH receptors to appear (Driancourt, 2001, Campbell et al., 2004, Stabenfeldt and Edquist, 1996). Oestrogen, however, has a negative feedback on FSH secretion which leads to a decrease in FSH levels by the selection stage (Driancourt, 2001). There is a switch from FSH to LH dependency as the follicle matures, however, it has been found that FSH alone can induce follicular growth to healthy pre-ovulatory size as long as FSH levels remain sufficiently high (Hunter et al., 2004).

During the selection process a dominant follicle appears while the other follicles of the cohort regress by atresia (Driancourt, 2001, Berisha and Schams, 2005). FSH induces the formation of LH receptors in the granulosa cells of mature follicles (Stabenfeldt and Edquist, 1996). The selected dominant follicle appears to be the first to develop LH receptors. LH receptor induction may be partially mediated by insulin-like growth factor (IGF)-1 (Driancourt, 2001). Increased inhibin and oestrogen produced by the selected follicle cause progressive negative feedback on FSH synthesis and release. FSH levels, at this point, are below the threshold for further follicular selection. The dominant follicle can now use LH to support its growth while smaller follicles have their development halted (Hunter et al., 2004, Stabenfeldt and Edquist, 1996). In sheep and cattle LH receptors can be detected in the granulosa layer of follicles 3.5 mm and 9 to 10 mm in diameter, respectively (Hunter et al., 2004).

During follicular dominance, the dominant follicle grows and matures while the subordinate follicles regress completely. LH is the key hormone involved in the final growth of the dominant follicle. LH induces androgen output by the theca cells stimulating oestrogen secretion by the granulosa cells (Driancourt, 2001). Oestrogen affects LH release by influencing both the hypothalamus and the anterior hypophysis. Through the stimulation of catecholaminergic neurons, oestrogen influences GnRH secretion. In the hypophysis, oestrogen increases the sensitivity of the pituitary to GnRH. As the dominant follicle matures and oestrogen production

increases, GnRH pulses increase in frequency resulting in the pre-ovulatory LH surge (Stabenfeldt and Edquist, 1996).

Even though LH receptors are present in the granulosa cells soon after dominant follicle selection, locally produced luteinization-inhibiting factors prevent the granulosa from becoming prematurely luteinized. After the LH surge, however, the granulosa becomes responsive to LH, begins to secrete progesterone and stimulates the production of intrafollicular substances which promote the rupture of the follicle and ovulation (Stabenfeldt and Edquist, 1996).

In domestic animals follicles continue to grow and develop during all phases of the oestrous cycle, including the luteal phase. In cattle, several dominant follicles develop during the luteal phase of the cycle at approximately 10-days intervals. Either the second or the third dominant follicle to develop during the luteal phase goes on to be the ovulatory follicle, depending on the timing of the initiation of regression of the CL. If CL regression occurs earlier, the second dominant follicle continues to develop through to ovulation; if later, the third dominant follicle becomes the ovulatory follicle (Stabenfeldt and Edquist, 1996). Bovine pre-ovulatory follicles reach a maximum of 16-22 mm in diameter while ovine ones range from 5-7 mm in dimater (Hunter et al., 2004).

The recurrent succession of recruitment, selection and dominance is termed as follicular wave. Sheep are shown to have 2 to 4 consecutive follicular waves per cycle (Driancourt, 2001, Berisha and Schams, 2005). The rising FSH at the time of luteal regression stimulates the development of antral follicles signalling the beginning of the follicular waves (Hunter et al., 2004). Very seasonal animals show no follicular waves during seasonal anoestrus and follicles do not exceed 3 to 4 mm in diameter. The wave pattern is slowly re-established during the transition towards the breeding season (Driancourt, 2001).

Although it is well established that ovarian function is regulated primarily by the pituitary gonadotrophins and their receptors, it is also evident that locally produced factors, such as steroid hormones, peptides and growth factors have essential modulatory roles in follicular development and ovulation (Berisha and Schams, 2005). Some factors like IGF-1 are required for all phases of pre-ovulatory

growth (Hunter et al., 2004) while others, like the growth hormone (GH), are known to be required only at certain stages of follicular development (Fortune, 2003).

The time covered by follicular development, ovulation, formation of the CL and atresia of the latter is termed oestrous cycle, and the interval between them averages 16 to 17 days in sheep (Hansel and McEntee, 1977, Bazer et al., 1994, Stabenfeldt and Edquist, 1996, Stellflug et al., 1997). The length of the follicular phase, however, corresponds to only 2 to 3 days (Hunter et al., 2004).

1.4 Luteal growth and development

CL development is characterized by a series of morphological and biochemical changes in the theca and granulosa layers of the ovulatory follicle. Its main function is to secrete progesterone which is essential for the maintenance of pregnancy. The CL is one of the few adult tissues that undergo development, function and regression during every cycle (Schams and Berisha, 2004).

1.4.1 Ovulation and CL formation

Ovulation in the ewe occurs 14 hours after the end of the LH surge and around 30 hours after the onset of oestrus (Stellflug et al., 1997, Bazer et al., 1994). CL formation consists of changes in the theca interna and granulosa cells of the pre-ovulatory follicle that occur after the pre-ovulatory LH surge (Berisha and Schams, 2005, Kaczmareck et al., 2005, Schams and Berisha, 2004). It has been suggested that the theca interna cells of the pre-ovulatory follicles differentiate into small luteal cells while the granulosa cells develop into large luteal cells. Small and large luteal cells are referred to as parenchymal cells and, together with fibroblasts and endothelial cells, form the basic structure of the CL (Berisha and Schams, 2005, Sawyer, 1995, Schams and Berisha, 2004).

Both small and large luteal cells synthesize and secrete progesterone. LH receptors can be found in early CL in both small and large luteal cells. LH stimulates progesterone production by small luteal cells while large luteal cells remain unaffected (Berisha and Schams, 2005, Sawyer, 1995). Although LH receptors are

found mainly in small luteal cells, large luteal cells are responsible for 80% of the total progesterone produced by the CL. Growth hormone (GH) receptors are mainly found on large luteal cells and GH is thought to be a more powerful stimulator of progesterone production in early CLs than LH (Schams and Berisha, 2004). Other factors are known to influence luteal progesterone and oxytocin secretion, such as the members of the IGF family. As the CL matures small luteal cells increase in numbers but not in size, whereas large luteal cells increase in size but not in number (Berisha and Schams, 2005, Sawyer, 1995).

Progesterone is essential for the maintenance of pregnancy and is produced by the CL, the placenta or both. In pigs and goats the maintenance of the CL is essential as placental progesterone production is very low. In cows, dogs, sheep and horses progesterone production during early pregnancy is extremely dependent on the presence of the CL but the placenta becomes the main source of of progesterone production as the pregnancy progresses. However, in sheep and primates, luteal production of progesterone continues throughout pregnancy even though placental production is dominant (Stabenfeldt and Edquist, 1996).

Tissue growth and development depend upon the establishment of adequate blood supply. The rapid growth of ovarian tissues must be equally accompanied by a rapid development of new blood vessels (Berisha and Schams, 2005, Kaczmareck et al., 2005, Reynolds et al., 2000, Redmer et al., 2001, Miyamoto et al., 2005).

The extent of angiogenesis within the CL reaches a maximum within 2 to 3 days after ovulation (Reynolds and Redmer, 1998, Berisha and Schams, 2005), and the early CL is resistant to endometrial prostaglandin 2α (PGF-2α). As the CL matures, the majority of steroidogenic cells establish contact with one or more capillaries, thus making the CL one of the most highly vascularised organs in the body. It is also at this time that the CL acquires the capacity to undergo luteolysis in response to endometrial PGF-2α (Sawyer, 1995).

Due to the absence of progesterone priming, a short-lived CL results from the first ovulation of the breeding season in ewes (Stellflug et al., 1997). Progesterone is known to affect CL functionality in an autocrine and/or paracrine way. During the early luteal phase the lack of progesterone exposure reduces further production of progesterone, oxytocin and luteal PGF-2α secretion. Luteal PGF-2α has a luteotropic

action as opposed to endometrial PGF-2α which has a luteolytic effect (Schams and Berisha, 2004). During the mid luteal phase progesterone exposure inhibits endometrial PGF-2α secretion thus preventing early luteal regression. It is thought that progesterone represses the onset of apoptosis in the CL by a progesterone receptor-dependent mechanism. Progesterone also stimulates synthesis of LH receptors in the CL, stimulating further progesterone production (Schams and Berisha, 2004).

It has also been suggested that abnormal luteal function is due to inadequate follicular development before ovulation (Southee et al., 1988b). Insufficient vascular support after ovulation and reduced number of luteal receptors are considered to be some of the causes of defective luteal function whilst the short life-span of the CL is attributed to premature PGF-2α release from the endometrium (Stellflug et al., 1997, Mann and Lamming, 2000).

Ovarian blood flow is highly correlated with progesterone concentration in systemic blood. Therefore, inadequate luteal function has also been associated with decreased luteal vascularity (Reynolds and Redmer, 1998, Bramley et al., 2005).

1.4.2 Luteolysis

Luteal regression is caused by episodic release of PGF-2α from the uterus which reaches the CL by a counter current system between the uterine vein and the ovarian artery. During early and mid luteal phases, progesterone inhibits endometrial PGF-2α secretion, however, by the late luteal phase the endometrium loses its sensitivity to progesterone and low receptor numbers are observed (Schams and Berisha, 2004).

Although progesterone inhibits luteolysis at an early stage it has an important role in the production of PGF-2α later in the cycle. The endometrium of ewes is stimulated by progesterone to increase phospholipids stores (arachidonic acid source) and cycloxygenase (COX) enzymatic activity necessary for the conversion of arachidonic acid to PGF-2α (Bazer et al., 1994). As concentration of progesterone declines, PGF-2α pulses increase in magnitude and frequency. The initiation of luteolysis involves oxytocin which engages in a positive feedback with PGF-2α (Hunter et al., 1989, Bazer et al., 1994, Berisha and Schams, 2005, Sawyer, 1995).

The normal CL contains a finite store of oxytocin. Luteal oxytocin synthesis and secretion are restricted to large luteal cells and achieve a maximum in the early luteal phase (Sawyer, 1995, Wathes and Lamming, 1995). However, endometrial oxytocin receptors must develop before the luteal oxytocin store is depleted (Wathes and Lamming, 1995). In sheep, endometrial oxytocin receptors are high between oestrus and days 4 to 5 of the cycle, low between days 5 and 13 and increase rapidly between days 14 and 16 (Bazer et al., 1994). The development of oxytocin receptors in the uterus is induced by oestrogen produced by the developing antral follicles (Stabenfeldt and Edquist, 1996).

Although oxytocin receptor levels are high during the early luteal phase they are under a negative inhibition by progesterone during the first 10 to 13 days of ovine (Bazer et al., 1994) and 16 to 17 days of bovine luteal phase (Mann and Lamming, 2000). Progesterone receptors are then in low numbers during day 12 and 14, allowing a rapid increase in oxytocin receptors in the late luteal phase. From day 14 (luteolytic period) ovine endometrium has low numbers of progesterone receptors and increasing oestrogen and oxytocin receptors (Bazer et al., 1994). Oxytocin then binds to its receptor and triggers the release of PGF-2α (Bazer et al., 1994). About 5 pulses of PGF-2α occur every 25 hours between days 14 and 17 or until the CL is depleted of its oxytocin store (Bazer et al., 1994, Ronayne et al., 1990).

Functional luteolysis involves the decline in progesterone secretion and begins 3 to 6 hours after the initiation of PGF-2α release (Stabenfeldt and Edquist, 1996). The main luteolytic action of PGF-2α is a rapid decrease in luteal blood flow (Berisha and Schams, 2005, Miyamoto et al., 2005, Sawyer, 1995, Reynolds and Redmer, 1998, Nett et al., 1976) with an increase in the number of other cell types such as immune cells and fibroblasts (Al-zi'abi et al., 2003). PGF-2α, however, initially stimulates a temporary vasodilatation of the local blood vessels. This acute increase in blood flow then triggers the cascade of luteolysis by stimulating the release of vasoconstrictive peptides and inflammatory cytokines (Berisha and Schams, 2005, Miyamoto et al., 2005) The regression of the CL is usually complete within 24 to 48 hours in large domestic species (Stabenfeldt and Edquist, 1996).

The high correlation between systemic levels of progesterone and blood flow to the luteal ovary suggests that blood flow may play an important role regulating

luteal function (Nett et al., 1976).

1.5 Hormone induced ovulation

A great number of methods have been developed to stimulate and induce cyclic activity in ewes. The methods may vary in their approach but all of them involve the manipulation of follicular growth and luteal life-span (Husein and Kriddli, 2003). The use of rams and/or hormones to induce oestrus and ovulation facilitates out-of-season breeding, the use of artificial insemination and embryo transfer. Unfortunately, ovulatory therapy can result in the formation of dysfunctional CL and progesterone deficiency is a possible factor involved in pregnancy failure (Murdoch and Van Kirk, 1998, Pearce et al., 1987).

1.5.1 GnRH induced ovulation

Pulsatile secretion of GnRH is responsible for the pulsatile release of LH from the pituitary which results in the last stages of follicular development and subsequent ovulation. Large doses of GnRH given to seasonally anoestrous animals result in an immediate pre-ovulatory type LH surge followed by a downregulation of LH release thereafter (McLeod et al., 1982b, Stabenfeldt and Edquist, 1996). However, small but frequent doses of GnRH result in a sustained elevation of circulating LH sufficient to induce the final stages of follicular development and ovulation in approximately 100% of treated animals (Stabenfeldt and Edquist, 1996, McLeod et al., 1983, McLeod et al., 1982a, McLeod et al., 1982b, McNatty et al., 1982, Southee et al., 1988b, Southee et al., 1988a). In essence, the small-dose multiple injections of GnRH given to stimulate an increase in LH pulses are designed to promote the pattern of LH release seen during the follicular phase of the oestrous cycle.

The induction of ovulation with GnRH in seasonally anoestrous ewes is often associated with inadequate luteal function (Southee et al., 1988a, Southee et al., 1988b, McLeod et al., 1982b). The lack of normal luteal function in such animals has been attributed to inadequate follicle development before the induction of ovulation (McLeod et al., 1982b). However; when progesterone pre-treatment is given as early

as 3-5 days before the start of GnRH treatment, even if it only raises plasma levels for about 30 h, the subsequent CL gives rise to a pattern of progesterone secretion typical of a normal luteal phase (Southee et al., 1988b, Pearce et al., 1987). Progesterone treatment prior to GnRH treatment results in approximately 100% normal luteal function against 20 to 40% of non-primed animals (McLeod et al., 1982b, Bartlewski et al., 2001, Hunter et al., 1988, Husein and Kriddli, 2003, Haresign et al., 1996, Southee et al., 1988a).

Oestrus occurs concomitant with the pre-ovulatory LH surge in all ewes pre-treated with progesterone. The LH surge in these animals, as in cyclic ewes, occurs due to oestrogen positive feedback and not due to the direct effect of multiple low-dose injections of GnRH. Ewes primed with progesterone have an LH surge significantly later than the non-primed animals (39 and 20 hours after the start of the GnRH treatment, respectively), allowing their follicles to be exposed to LH secretion for longer. A reduced exposure to LH has been suggested as one of many explanations for the formation of defective CL (McLeod et al., 1982b). Progesterone priming, however, has no effect on the time of onset, duration or amplitude of the pre-ovulatory LH surge (Legan et al., 1985).

The delayed occurrence of the LH surge does not seem to be essential for normal luteal function as some of the non-primed ewes are capable of producing a functional CL even though their LH surge occurs at the same time as that of the animals that developed defective CLs. This suggests that progesterone may have a direct effect on the ovary, either by synchronizing follicular development before the start of the GnRH treatment or by possibly increasing responsiveness of the developing follicles to gonadotrophins (Southee et al., 1988b). When given intravenously, a GnRH bolus is able to produce an immediate pre-ovulatory LH surge (Hunter et al., 1988). When this was used to avoid any delay in the pre-ovulatory surge between progesterone primed and non-primed animals it indicated that the extended exposure of pre-ovulatory follicles to episodic LH secretion prior to the pre-ovulatory LH surge is not a pre-requisite for the maintenance of the CL after ovulation (Haresign et al., 1996).

The exact mechanism of action of progesterone priming on subsequent normal luteal function is still not clarified. However, progesterone priming does not significantly change the pattern of LH secretion during GnRH treatment, nor the

duration or amplitude of the pre-ovulatory LH peak. Progesterone priming does not have any effect on the timing, duration or amplitude of the LH surge, supporting the concept that progesterone priming does not promote normal luteal function by altering patterns of LH secretion during the pre-ovulatory period (Haresign et al., 1996). Although much has been suggested it is still not clear how progesterone influences the development of functional CLs.

1.5.2 Defective CL

Defective luteal function has been attributed as one of many causes of infertility (Yaniz et al., 2008) and is of relatively common occurrence in livestock reproduction. At puberty and following post-partum and seasonal anoestrous, the first oestrous cycle observed in the majority of animals is often of short duration with a short lived CL (Mann and Lamming, 2000, Crowe, 2008, Bulman and Lamming, 1978).

Most cyclic and progesterone primed seasonally anoestrous ewes induced to ovulate with multiple GnRH injections are capable of showing normal luteal function. Normal luteal function is determined by an elevation in plasma progesterone concentration for at least 9 days; the progesterone rise should start within 3 to 4 days of the LH surge and reach a maximum of more than 1.5 ng/ml (Southee et al., 1988b, McLeod et al., 1982b). On the other hand, abnormal luteal function is defined as progesterone concentrations below 1 ng/ml during the same period in animals in which luteolysis is blocked by hysterectomy (Southee et al., 1988b).

The abnormal CLs developed by entire non-primed anoestrous ewes show a transient increase in progesterone concentration (0.5 – 1 ng/ml) between day 1 and 2, similar to those of cyclic ewes or progesterone primed ewes, before returning to base line levels by day 6 after ovulation (Hunter et al., 1988, Southee et al., 1988b). This transient rise in progesterone, however, is enough to ensure a subsequent normal function of the next cycle CL (Southee et al., 1988b).

Although most animals that do not receive progesterone pre-treatment develop abnormal CL, these CL are morphological indistinguishable from normal CL up to

day 4 but differences become apparent on day 5 after ovulation (Southee et al., 1988b, Hunter et al., 1988). While functional CL increase in weight, defective CLs either stop growing altogether or show signs of regression, and also show disorganized morphological appearance and significantly lower progesterone content (Hunter et al., 1989). It has been suggested that the defective production of progesterone is due to inadequate supply of substrate. This, however, may be a consequence of luteal regression rather than its cause (Hunter et al., 1988). Luteal functionality is related to the existing ratio between small and large luteal cells. Although abnormal CL seem to have less overall amount of luteal cells (Bramley et al., 2005) the small:large luteal cells ratio remains the same as that found in functional CLs (Hunter et al., 1988).

By day 4 after ovulation, defective CL show reduced number of gonadotrophin binding sites and an increased sensitivity to luteolytic agents (Hunter et al., 1988, Southee et al., 1988b). It is important to note that the reduced number in gonadotrophin binding sites in defective CL is not due to a decrease in receptor concentration but rather a failure of the receptor numbers to increase, suggesting that the lack of gonadotrophin receptors is not the fundamental cause of luteal abnormality (Hunter et al., 1988). Abnormal CL also show significantly lower vascular surface (Bramley et al., 2005) and appear pale in comparison to dark pink normal CL (Hunter et al., 1988). The number of CL developed per animal also does not influence luteal functionality: in animals with more than one ovulation, either both are normal or both are abnormal (Southee et al., 1988b).

Another luteal defect observed in non-primed ewes was a lack of follicular development at the time of ovulation. It has been suggested that inadequate priming of the pre-ovulatory follicle by oestradiol results in lutein abnormality of granulosa cells (Murdoch and Van Kirk, 1998).

Regression of abnormal CL is thought to be PGF-2α dependent as that of a normal CL, as non-primed hysterectomized animals show persistent but defective CL (Mann and Lamming, 2000). Oxytocin binding sites are present and PGF-2α is released in luteolytic pulses after 2 to 3 days of slightly elevated progesterone in contrast to 7 to 10 days of cyclic ewes (Hunter et al., 1989). The premature luteolysis observed in defective CL does not result from a direct effect of the lack of

progesterone action in the uterus but rather from an indirect effect mediated through an inadequate level of pre-ovulatory oestrogen production. It has been proven that in the absence of progesterone, oestrogen has the ability to cause a decrease in endometrial oxytocin receptor numbers. Reduced secretion of oestrogen by the pre-ovulatory follicle in non-primed animals, therefore, leads to an impaired inhibition of endometrial oxytocin receptors and binding of oxytocin to these receptors results in the premature luteolytic episodes of PGF-2α by the uterus leading to early luteolysis (Mann and Lamming, 2000).

It has also been suggested that the premature luteolysis that occurs in the abnormal CL is due to an increased sensitivity of the tissue to luteolysins and/or inadequate protection (Hunter et al., 1988, Southee et al., 1988a). However, the reasons for this possible increased sensitivity in abnormal CL have yet to be fully determined. The regression of the short-lived CL is dependent on uterine PGF-2α release, however, the abnormality of the CL is not. Defective luteal function, therefore, is not due to premature regression but possibly inadequate follicular development and inadequate microvasculature of the pre-ovulatory follicle and subsequent CL (Southee et al., 1988a).

1.6 Angiogenesis

1.6.1 Introduction

Two blood vessel development processes have been identified in animals and humans, vasculogenesis and angiogenesis.

Vasculogenesis consists of the initial differentiation of embryonic mesenchymal cells into hemangioblasts, and angiogenesis is the formation of new blood vessels by sprouting from an already existent endothelium (Ferrara et al., 1992, Berisha and Schams, 2005, Kaczmareck et al., 2005). Whereas vasculogenesis is restricted to embryonic development, angiogenesis continues to operate throughout life when new vascularisation is required. Although angiogenesis can be present in pathological processes (such as tumours) in any body tissue, the vascular network of the adult is

relatively stable and physiological angiogenesis during adult life is mainly restricted to wound healing and the female reproductive tract (Korpelainen and Alitalo, 1998).

The process of angiogenesis is essential for tissue growth and development and has been demonstrated to be a critical component during follicular and luteal function and development (Tamanini and De Ambrogi, 2004). Angiogenesis consists of 3 main steps: 1) degradation of the basement membrane of a local blood microvessel after the release of proteases, 2) migration of endothelial cells and 3) proliferation of endothelial cells (Ferrara et al., 1992, Redmer et al., 2001, Berisha and Schams, 2005, Kaczmareck et al., 2005).

Vascular remodelling involves establishment of directional flow and association with mural cells such as pericytes and vascular smooth muscle cells. When new blood vessels are formed, pericytes migrate from existing arterioles along the side branch of the preformed endothelial plexus leading to a progressive coverage of the vascular tree (Benjamin et al., 1998). Pericytes can be found at and in front of the advancing tips of endothelial cell sprouts, possibly supporting endothelial cell outgrowth (Nehls et al., 1992). Initially, the vascular density exceeds the nutritional requirements of the tissue but is later adjusted by regression of unnecessary vessels. It has been suggested that after the acquisition of a pericyte coating no further vascular remodelling takes place (Benjamin et al., 1998).

Pericytes are surrounded by a basement membrane and intimately associated with endothelial cells (Benjamin et al., 1998). They are long, slender, polymorphic cells with an elongated body from which branches arise. Pericytes incompletely surround endothelial cells and the microvascular basement membrane of capillaries. The same basal lamina that envelops cell bodies and cytoplasmatic processes of endothelial cells also envelops those of pericytes except for where these two cells make direct contact. Pericyte-endothelial cell contact are by adhesion plaques and gap junctions, possibly making up a structural mechanism in which they respond to signals generated by other cells. Pericytes express typical contractile cell protein like that found in vascular smooth muscle cells. A gradual transition can be observed from the pericytes to smooth muscle cells, indicating that pericytes are precursors of the latter (Diaz-Flores et al., 1991). It is said that pericytes are a transition between vascular smooth muscle cells and non-muscle cells (Nehls and Drenckhahn, 1991). Several

functions have been attributed to pericytes, including: contractibility and permeability regulator, integrity maintenance, endothelial cell growth modulator and cell progenitor (Diaz-Flores et al., 1991, Benjamin et al., 1998).

1.6.2 Angiogenesis and folliculogenesis

Adequate ovarian vasculature is essential for follicular development and ovulation (Shimizu et al., 2007). As the follicles mature, primary follicles develop into secondary follicles containing two layers of cells: the theca layer with its own vascular supply and the granulosa layer, which is avascular (Tamanini and De Ambrogi, 2004). The establishment of the theca layer precedes angiogenesis, and the first endothelial cells become detectable when more than four granulosa layers become established (Wulff et al., 2001a). These newly formed ovarian blood vessels provide an increasing supply of gonadotrophins, growth factors, oxygen, steroid precursors, as well as other substances to the growing follicle. In the theca layer, blood vessels increase in number and size as the follicle develops but do not penetrate the basal membrane separating the theca interna from the granulosa cell layer (Kaczmareck et al., 2005).

As the antrum develops, the theca layer acquires two capillary networks located in the theca interna and extena. To support the increasing blood flow during follicular development, theca capillaries undergo angiogenesis and vasodilatation (Tamanini and De Ambrogi, 2004, Hunter et al., 2004).

Although angiogenesis takes place in the theca layer, the granulosa cells are responsible for significant production of angiogenic factors, which will act on the theca layer (Tamanini and De Ambrogi, 2004). In pigs, medium sized antral follicles show two concentric vascular networks: an inner network near the basal membrane and an outer network within the theca externa (Mattioli et al., 2001). It has been hypothesised that angiogenic factors accumulate in the follicular cavity, diffuses to the theca compartment and create an angiogenic gradient attracting blood vessels to the granulosa layer. The basal membrane, however, proves to be a physical barrier for this migration, and these blood vessels develop within the theca interna in strict contact with the basal membrane. This inner capillary network represents the main

source of nutrients for the avascular granulosa. The persistence of this inner capillary network is directly dependent on VEGF accumulation in the follicular fluid and when levels are low (as in atretic follicles) it undergoes dramatic reduction (Mattioli et al., 2001).

The acquisition of adequate vascular supply is said to be a limiting step in the selection and maturation of the main follicle (Hunter et al., 2004), and the development of a microvasculature is essential for the delivery of hormones and nutrients to developing follicles prior to ovulation and CL development (Tamanini and De Ambrogi, 2004).

Dominant follicles have a higher percentage of blood vessels in the theca layer than smaller antral follicles within the same ovary or in the contralateral ovary (Acosta et al., 2003). There is a positive relationship between blood flow in the follicular wall and plasma oestrogen and LH concentrations. The first detectable increase in plasma oestrogen concentrations is coincident with an increase in vascularisation in the follicular wall (Acosta et al., 2003, Hunter et al., 2004). Dominant follicles have significantly higher oestrogen:progesterone ratio in their follicular fluid than non-dominant follicles (Jiang et al., 2003). Follicles with high oestrogen:progesterone ratio present well developed capillaries with active angiogenesis and a more uniform vessel network distribution than follicles with low oestrogen:progesterone ratio (atretic) (Jiang et al., 2003). It has been observed that increased vascularisation of individual follicles result in preferential delivery of gonadotrophins and therefore individual blood flow may play an essential role in the selective maturation of the pre-ovulatory follicle(s) (Zeleznik et al., 1981, Hunter et al., 2004, Kaczmareck et al., 2005, Acosta et al., 2003).

The onset of follicular vascularisation in the primate begins at the early secondary stage, increases during follicular development and decreases during follicular atresia (Wulff et al., 2001a). In humans, dominant follicle blood flow during the late follicular phase and just before the LH surge are the same. However, after the LH surge there is an increase in blood flow to the base of the follicle and a decrease in blood flow to the apex (Acosta et al., 2003). Vasoactive factors, besides regulating local vascularisation, also modulate local secretion of prostaglandin and steroid hormones (Acosta et al., 2003).

Around the time of the pre-ovulatory LH surge the basal membrane between the granulosa and theca interna layers of the developing follicles dissolves. The theca capillaries expand and sprout into the avascular granulosa layer to form a dense network (Shimizu et al., 2007).

1.6.3 Angiogenesis and luteogenesis

Soon after ovulation the collapsed follicle undergoes extensive changes including the luteinisation of the theca and granulosa cells, tissue growth and remodelling and increasing progesterone production. In order to meet these demands a well established vascular supply is essential (Robinson et al., 2007).

The early CL consists of tissue infolding formed from the collapsed follicular wall. The early CL also has a central cavity which is the remnant of the follicular antrum. Most of the blood vessels of the early CL are present at the base of these infoldings which probably derive from the theca layer and are composed of connective tissue. Towards the end of the early luteal phase, the infoldings expand towards the centre of the CL, obliterating the central cavity, and the base of these infolding expansions become less prominent but give the CL a lobulated aspect and continue to contain the majority of the microvessels. However, numerous capillary sprouts can be seen invading the luteinised derived parenchyma (Zheng et al., 1993).

The early luteal phase is characterized by the invasion of capillaries derived from the theca layer and rapid proliferation of endothelial cells (Zheng et al., 1993). Endothelial cells represent 85% of the proliferating cells found in the early ovine CL (Reynolds et al., 1994, Schams and Berisha, 2004). The sprouting endothelial cells invade the cavity of the newly formed CL and establish a vascular bed between the luteal cells, which will result in a dense network of fully differentiated capillaries by the mid-luteal phase. Luteal blood flow gradually increases from days 2 to 5 after ovulation. The high proliferation of endothelial cells in the early luteal phase is responsible for the increased vascularity of the mature CL. The presence of a dense capillary network is essential for optimal delivery of nutrients and progesterone precursors and for the secretion of progesterone itself (Al-zi'abi et al., 2003, Schams and Berisha, 2004).

During the mid luteal phase a dense network of capillaries can be observed surrounding the luteal parenchyma with many of the parenchymal cells being adjacent to at least one capillary. Vascular distribution during this stage appears to be heterogeneous, with some areas showing high capillary density and others low capillary density (Zheng et al., 1993).

At this stage endothelial cells represent more than 50% of the cells present in the ovine CL (Schams and Berisha, 2004, Reynolds et al., 1994). During the mid luteal stage the area and density of microvessels are significantly greater than those observed in the early luteal phase (Al-zi'abi et al., 2003).

The late luteal phase is characterized by the regression of the vascular network, an increase in connective tissue and the loss of luteal parenchymal cells. Although a significant reduction in the number of capillaries can be seen, large microvessels remain proeminent (Zheng et al., 1993). By the late regression stage the extensive vascular network observed during the mid luteal phase has regressed (Al-zi'abi et al., 2003).

In equine CL, total cell proliferation index is highest in the early luteal phase and declines by 70% during the mid-luteal and early regression phase (Al-zi'abi et al., 2003). However, during the late regression phase a significant increase in the proliferation index can be observed when compared to early regression. Endothelial cell proliferation follows this index very closely, showing highest proliferation rate during the early luteal phase and significantly decreasing in number by the mid-luteal phase and early regression. However, during the late regression phase very few endothelial cells undergo proliferation, suggesting that the increase in the total cell proliferation index at this time is probably due to other cell types such as leucocytes and fibroblasts. During the early and mid stages of luteal development; endothelial cells correspond, respectively, to approximately 95% and 80% of the total cell proliferation taking place. However, these levels significantly decrease to 33% by the early and to 6% by the late stages of luteal regression (Al-zi'abi et al., 2003).

Sheep CL exhibit high rates of cellular proliferation from days 2 to 12 of the oestrous cycle (day 0 = oestrus). Both parenchymal (steroidogenic) and non-parenchymal (endothelial cells, fibroblasts, smooth muscle cells) luteal cells proliferate throughout the oestrous cycle. Even though functional differentiation of

the CL is completed by day 8, luteal growth and cellular proliferation continues up to day 12 after ovulation. The rate of tissue growth is greater than that of the fastest growing tumour. The estimated number of luteal cells proliferating increases 3.5 fold between day 2 and 4, remains high through to day 12 and decreases between day 12 and 15 after ovulation. By day 8, a 14 fold increase can be observed in the number of luteal cells, and steroidogenic cells correspond to the minority of proliferating cells. From day 8 to 12, even though the proliferation rate remains high, the number of luteal cells only increases by 1.3 fold, and on day 15 the number of luteal cells is similar to those observed on day 12 after ovulation (Jablonka-Shariff et al., 1993). Plasma progesterone concentration has a similar profile to that of luteal cell content, with an increase from early to mid luteal phase. The increase in plasma progesterone and content are associated with increased luteal weight (Jablonka-Shariff et al., 1993).

Although there is no doubt that pituitary gonadotrophins are essential for normal ovarian function, factors that may regulate the exposure of ovarian cells to the gonadotrophins may play an important role in follicular maturation and CL development (Ravindranath et al., 1992). As mentioned previously, pre-ovulatory follicles have a higher blood supply and gonadotrophin exposure than smaller follicles and angiogenic factors are thought to indirectly regulate follicular selection by regulating blood vessel formation.

Many factors have been implicated as positive regulators of angiogenesis, including the acid Fibroblast Growth Factor (aFGF), basic FGF, Transforming Growth Factor (TGF), Vascular Endothelial Growth Factor (VEGF) and Angiopoietins (Ferrara et al., 2003b, Kaczmareck et al., 2005, Bramley et al., 2005). Even though individual factors are nevertheless important, angiogenesis seems to depend on an interaction between these factors acting at different levels of blood vessels formation and maturation (Tamanini and De Ambrogi, 2004).

1.7 The VEGF family

1.7.1 Molecular biology of VEGF

The VEGF family comprises of a group of growth factors denoted VEGF-A (referred to as VEGF), Placenta Growth Factor (PIGF), VEGF-B, VEGF-C , VEGF-D and VEGF-E, a VEGF-like protein encoded by the Orf virus (Ferrara, 2001). Deletion of both or a single VEGF allele is lethal during foetal development as is overexpression (Tamanini and De Ambrogi, 2004, Scheidegger et al., 1999).

This family of growth factors is responsible for regulating the growth and differentiation of multiple components of the vascular system. VEGF-A is responsible for regulating blood vessel angiogenesis, while VEGF-C and VEGF-D regulate lymphatic angiogenesis (Ferrara et al., 2003b). VEGF has 3 main roles in angiogenesis: (1) stimulating endothelial cell proliferation and migration, (2) maintaining immature blood vessel viability and (3) facilitating the process of pericytes recruitment (Benjamin et al., 1998).

VEGF also acts as a mitogen for vascular endothelial cells and there is strong evidence that it is also essential for the survival of these cells by inducing the expression of anti-apoptotic proteins in human endothelial cells (Ferrara, 2001). VEGF is also known as vascular permeability factor due to its ability in promoting vascular leakage and the formation of fenestrations in certain endothelial cells inducing protein extravasion necessary for endothelial cell growth (Ferrara et al., 2003b, Hunter et al., 2004). VEGF permeability potency is 50,000 fold higher than histamine (Tamanini and De Ambrogi, 2004) and its permeability is thought to allow large molecules, like LH, to penetrate the endothelium and reach the theca cells (Wulff et al., 2001a).

Although VEGF seems to act mainly on endothelial cells, it has also been reported that, *in vitro*, VEGF has a mitogenic effect on some non-endothelial cells such as retinal pigment epithelial cells and pancreatic duct cells (Ferrara, 2001). Furthermore, VEGF promotes growth and multiplication of vascular endothelial cells and marrow-derived cells (Ferrara et al., 2003b, Hunter et al., 2004).

When cells are exposed to low oxygen or nutrients, as in growing tumors, VEGF secretion rate is enhanced (Scheidegger et al., 1999, Neulen et al., 1998). Tumour cells also produce bFGF that further stimulates VEGF production in vascular smooth cells and endothelial cells. Fibroblasts are also stimulated to produce VEGF when serum leaks from blood vessels (Scheidegger et al., 1999). On the other hand, natural vascular pruning is induced by transient hyperoxia around the newly formed arterial vasculature. Hyperoxia causes excessive regression of capillaries from the immature vasculature. This vessel regression occurs due to down regulation of VEGF beyond the required level to sustain newly formed vessels. Hyperoxia refractoriness is determined by pericyte coating of the vessel. Local VEGF injection accelerates the process of pericyte coating and completely prevents vessel regression. This pericyte coating prevents deleterious vessel regression in mature animals in occasional cases of transient fluctuations of tissue oxygen. It has been suggested that in some tissues, like the retina, pericytes are unlikely to play a role in initial formation of blood vessels but, instead, appear to influence maturation and remodelling of these vessels (Benjamin et al., 1998).

VEGF stimulates nitric oxide (NO) synthase expression in endothelial cells, thus increasing NO synthesis. Initially NO has a positive feedback on VEGF expression by vascular smooth muscle cells and pericytes (Beckman et al., 2006), however, large quantities of NO markedly reduces VEGF production. Basic FGF helps stimulate angiogenesis by blocking the negative feedback that NO has on VEGF production (Grasselli et al., 2002).

1.7.2 VEGF isoforms

VEGF is a glycoprotein encoded by a single gene (Wiesmann et al., 1997, Scheidegger et al., 1999) and alternative splicing of the VEGF gene results in the formation of different isoforms that vary according to the number of amino acids present after signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$) (Ferrara et al., 2003b, Houck et al., 1991).

Human VEGFs are longer by one residue in the N-terminal region than most mammalian VEGFs, so human $VEGF_{121}$ would correspond to mice $VEGF_{120}$. Canine

VEGF amino acid sequence displays only 4.8% changed residues compared to the human sequence. Based on these sequences it has been assumed that canine and human VEGFs are structurally almost identical. None of the differences seem to be located in positions thought to be relevant for the biological action of VEGF. It also has been demonstrated that the biological properties of canine VEGF are identical to those of humans (Scheidegger et al., 1999).

The human VEGF gene comprises 8 exons (Tischer et al., 1991). Exons 1-5 of the VEGF gene encode the domain responsible for the recognition of the receptors *fms*-like tyrosine kinase (Flt-1 or VEGFR-1) and fetal liver kinase-1/kinase insert domain-containing receptor (Flk-1/KDR or VEGFR-2) and are present in all VEGF isoforms (Keyt et al., 1996). The amino acids encoded by exon 8 can also be found in all VEGF variants. The difference between the various VEGF isoforms is therefore due to the presence or absence of the amino acids encoded by exons 6 and 7 (Poltorak et al., 1997, Tischer et al., 1991). The peptide encoded by exon 7 seems to affect receptor recognition of the different isoforms. $VEGF_{121}$ (lacking this peptide) recognises a single VEGF receptor (VEGFR-2) in endothelial cells while $VEGF_{165}$ (which contains exon 7 encoded peptide) binds to VEGFR-2 but recognises two other receptors (Poltorak et al., 1997). Endothelial cell proliferation and angiogenesis are not dependent on the presence of either exon 6 or 7 and seem to be more associated with limited VEGFR-2 binding (Poltorak et al., 1997).

The most abundant product of the VEGF gene, in humans, is the mRNA transcript encoding $VEGF_{165}$, with the exception of the placenta where $VEGF_{121}$ transcript is more abundant (Ferrara et al., 1992).

$VEGF_{121}$ lacks exons 6 and 7 (Poltorak et al., 1997) and is a 34 to 36 kDa homodimer peptide which is weakly acidic, fails to bind to heparin and can be found in its freely diffusible form (Poltorak et al., 1997).

Purified $VEGF_{165}$ has a molecular weight of approximately 46 kDa and dissociates upon reduction into two identical 23 kDa subunits (Tischer et al., 1991, Houck et al., 1992). $VEGF_{165}$ contains the amino acids encoded by exon 7 (Poltorak et al., 1997) and is a basic heparin-binding protein of which, although secreted freely, a significant fraction remains bound to the cell surface and extracellular matrix (ECM). In comparison to $VEGF_{121}$, the 44 amino acid insertion found in $VEGF_{165}$

contains many basic residues, thus giving it a more basic characteristic and an isoelectric point (pI) of approximately 8.5. The same can be further observed with $VEGF_{189}$ since 12 of the 24 amino acids inserted are basic residues. $VEGF_{189}$ contains both exons 6 and 7 encoded peptides (Poltorak et al., 1997), adding 24 amino acids at position 116 (Houck et al., 1992).

Recently, other VEGF isoforms have been reported, including $VEGF_{145}$ and $VEGF_{183}$ (Ferrara, 2001). $VEGF_{145}$ contains exons 1-6 and exon 8 with a total of 145 amino acids. This isoform is usually found as a homodimer of approximately 41 kDa but small amounts of monomeric forms can be found. $VEGF_{145}$ dimers can be dissociated into monomers upon reduction. $VEGF_{145}$ has a heparin binding affinity similar to $VEGF_{165}$, it induces proliferation of human umbilical vein-derived endothelial cells (HUVECs) but $VEGF_{165}$ is 6-fold more effective. It has also been shown that this isoform is capable of inducing angiogenesis *in vivo*. $VEGF_{145}$ binds to VEGFR-2 but not to other receptors. The presence of exon 6, despite conferring heparin-binding properties, does not seem to be sufficient to enable efficient binding of $VEGF_{145}$ to those receptors. Several cell lines do not express $VEGF_{145}$ mRNA, indicating that this isoform expression may be more restricted than that of the other isoforms (Poltorak et al., 1997).

In 1991, a new isoform, $VEGF_{206}$, was found. This isoform comprises a 41 amino acid insertion relative to $VEGF_{165}$ and the highly basic 24 amino acid insertion found in isoform 189 (Houck et al., 1991). $VEGF_{206}$ is identical to $VEGF_{189}$ with an additional 17 amino acid sequence (Houck et al., 1992). Like $VEGF_{189}$, $VEGF_{206}$ is more basic than acidic and binds to heparin with greater affinity than the $VEGF_{165}$, isoform thus suggesting that exon 6 might contribute to this binding. These variants are almost completely sequestered in the ECM (Ferrara, 2001). Once released from the cell membrane or extracellular matrix, $VEGF_{189}$ has been proven to stimulate endothelial cell growth *in vitro* (Houck et al., 1992). $VEGF_{206}$ is predominantly cell-associated and secreted only in very low levels despite the presence of signal peptide in the same concentration as for $VEGF_{121}$ and $VEGF_{165}$ (Houck et al., 1991).

Isoforms 121, 145 and 165 are secreted by producing cells and found free in the medium (Poltorak et al., 1997). A significant percentage of $VEGF_{165}$ (Houck et al., 1992) and $VEGF_{145}$ are also found bound to the ECM (Poltorak et al., 1997).

However, $VEGF_{145}$ binds to the ECM by a mechanism independent of ECM-associated heparin sulphates. It seems that simultaneous presence of both exons 6 and 7 is required for efficient binding of VEGF to cell surfaces, whereas the presence of each of these exons on its own does not confer this property (Poltorak et al., 1997).

VEGF165, 189 and 206 are found mainly in the extracellular matrix forming a complex with heparin sulphate-containing proteoglycans (Wiesmann et al., 1997). (Poltorak et al., 1997).

These large isoforms bind to heparin containing compounds on the cell surface or in the extracellular matrix. Heparin competes with $VEGF_{189}$ for binding sites and can result in higher availability of unbound $VEGF_{189}$ isoform *in vitro*. Heparinase treatment also results in higher free $VEGF_{189}$ levels, confirming the affinity of this isoform for heparin containing compounds. Heparin sulphate-containing proteoglycans are the constituents of the ECM that are responsible for binding the growth factors in the matrix or cell surface. It is thought that cell surface associated heparin-like molecules are necessary for VEGF binding to its receptor on endothelial cells. The binding of VEGF to heparin sulphate proteoglycans could possibly have the function of providing a reservoir of biologically active VEGF available to endothelial cells (Houck et al., 1992) and may be the means of generating a high local gradient of active growth factor (Scheidegger et al., 1999).

Heparin-binding isoforms that are bound to cell surfaces or ECM can be released in soluble form by heparin or heparinase. The long isoforms may also be released by plasmin following cleavage at the COOH terminus. These findings suggest that VEGF can become available to endothelial cells as diffusible proteins ($VEGF_{121}$ and $VEGF_{165}$) or by following protease activation and cleavage of the longer isoforms (Ferrara, 2001). As $VEGF_{189}$ strongly interacts with heparin it has been suggested that this variant binds tightly to cell surface and ECM localized heparin sulphates. This was supported by the fact that heparin can dissociate $VEGF_{189}$ from ECM (Poltorak et al., 1997).

On the other hand, $VEGF_{165}$ interacts weakly with cell surface heparin sulphates. As $VEGF_{145}$ binds to heparin with the same strength as $VEGF_{165}$ it was expected also to show a weak interaction with ECM. However, it was observed that $VEGF_{145}$ binds

to ECM from corneal endothelial cells much better than $VEGF_{165}$. In addition, the $VEGF_{145}$ bound to ECM is biologically active, being able to induce endothelial cell proliferation (Poltorak et al., 1997).

Heparin binding ability does not seem to be related to receptor binding since $VEGF_{145}$ and $VEGF_{165}$ bind to heparin with the same affinity but differ in their ability to recognise the receptors as $VEGF_{145}$ recognizes only VEGFR-2, like $VEGF_{121}$, whilst $VEGF_{165}$ recognizes two other receptors (neurophilin-1 and -2) besides VEGFR-2 (Poltorak et al., 1997). There is also an inverse relationship between heparin affinity and diffusability. $VEGF_{121}$ has the lowest affinity to heparin and the highest bioavailability. Loss of heparin binding results in substantial loss of mitogenic activity for vascular endothelial cells (Ferrara, 2001).

Vascular permeability activity has been observed in all isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$); however, endothelial cell mitogenic activity has only been apparent in cell cultures treated with $VEGF_{121}$ and $VEGF_{165}$ (Houck et al., 1991). $VEGF_{121}$ and $VEGF_{165}$ promote angiogenesis, induce permeabilization of blood cells and proliferation of vascular endothelial cells. It is suggested that $VEGF_{189}$ may induce endothelial cell proliferation. $VEGF_{145}$ is the main isoform expressed by cell lines derived from carcinomas of the female reproductive system (Poltorak et al., 1997).

1.7.3 VEGF Receptors

VEGF binds to two tyrosine kinase receptors: VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed almost exclusively on endothelial cells (Korpelainen and Alitalo, 1998) and are very important in embryo blood vessel formation. It has been reported that VEGF binds to both receptors with high affinity. A higher affinity has been observed for VEGFR-1, however, there is evidence that VEGFR-2 is the key signalling receptor for VEGF (Ferrara, 2001). Although VEGFR-1 exhibit higher affinity for some VEGF isoforms when compared to VEGFR-2, its function in the adult vasculature remains poorly understood (Gille et al., 2001).

Both receptors are composed of seven immunoglobulin (Ig)-like domains in the extracellular domain, a single transmembrane region and a tyrosine kinase (TK) domain which is interrupted by a kinase insert domain. VEGF binds to the second Ig-like domain of the receptors and deletion of this domain abolishes VEGF binding completely (Ferrara, 2001).

VEGFR-1 and –2 have different signal transduction properties. VEGFR-2 undergoes strong ligand-dependent tyrosine phosphorylation in endothelial cells while VEGFR-1 reveals weak responses. VEGF mutants that bind selectively to VEGFR-2 are able to induce normal mitogenesis and chemiotaxis in endothelial cells, angiogenesis and permeability. In contrast, VEGFR-1 selective VEGF mutants are devoid of such activities. PIGF binds with high affinity to VEGFR-1 and not VEGFR-2 and lacks direct mitogenic or permeability-enhancing properties (Ferrara, 2001, Gille et al., 2001). However, high concentrations of PIGF that are expected to saturate VEGFR-1 sites are able to potentiate VEGF activity *in vitro* and *in vivo*. These findings lead to the hypothesis that VEGFR-1 may not be the main signalling receptor for VEGF but a "decoy" receptor able to regulate VEGF activity by making the ligand less available for VEGFR-2 (Ferrara, 2001).

It has been proven that VEGFR-1 is essential for the organization of embryonic vasculature but not essential for endothelial cell differentiation (Fong et al., 1995). VEGFR-1 is said to be involved in organization of endothelial cell morphology (Scheidegger et al., 1999) and critical for VEGF induced tube formation (Tamanini and De Ambrogi, 2004). Mice embryos lacking VEGFR-1 show differentiation of endothelial cells with disruptions at later stages of vasculogenesis resulting in thin-walled vessels of large diameter and embryo death at 9 days (Folkman and D'amore, 1996). VEGFR-1 contains a repressor sequence in its juxtamembrane region that inhibits VEGF dependent signal transduction. Deletion of such repressor confers VEGFR-1 the ability to mediate VEGF dependent endothelial migration. These results suggest that VEGFR-1 plays a role in angiogenesis by binding to the ligand rather than transducing a signal after the binding (Ferrara, 2001). VEGFR-1 can be found as a full-length membrane–bound receptor and as a soluble truncate form containing the extracellular VEGF-binding domain but lacking the transmembrane segment and the kinase domain. This truncated form arises through alternative

splicing and it is thought to negatively control VEGF action. In the majority of tumours VEGFR-1 is overexpressed 2 to 6-fold when compared to healthy tissues. Over expression of the truncated form might be useful as an anti-angiogenic protein in selected tumours (Scheidegger et al., 1999).

VEGFR-2 is expressed by endothelial cells and its phosphorylation stimulates mitogenesis and migration of endothelial cells while VEGFR-1 has no significant effect (Gille et al., 2001, Scheidegger et al., 1999, Tamanini and De Ambrogi, 2004).

Mice embryos deficient in VEGFR-2 die during early embryological development (day 8.5 – 9.5) and showed no blood islands or organized blood vessels thus proving the importance of VEGFR-2 in embryo vasculogenesis (Shalaby et al., 1995). The lack of VEGFR-2 is thought to interfere with the differentiation of endothelial cells (Folkman and D'amore, 1996). It has been proven that VEGFR-2 is effective in inducing *in vivo* angiogenesis while VEGFR-1 was unable to do so. Therefore, only VEGFR-2 appears capable of promoting angiogenesis *in vivo*. It has been suggested by some authors that VEGFR-2 is the sole mediator of VEGF induced migration, angiogenesis and permeability in endothelial cells (Gille et al., 2001).

Some VEGF isoforms also bind selectively to two neurophilin type 1 transmembrane receptors, NP-1 ($VEGF_{165}$) and NP-2 ($VEGF_{165}$ and $VEGF_{145}$) (Tamanini and De Ambrogi, 2004). Neurophilin-1 is an isoform specific VEGF receptor that presents $VEGF_{165}$ to its signalling receptor in a manner that enhances the effectiveness of the signal transduction cascade. $VEGF_{121}$ fails to bind to this receptor thus partially explaining its lower bioactivity when compared to that of $VEGF_{165}$. Inhibition of $VEGF_{165}$ binding to neuropilin-1 inhibits its binding to VEGFR-2 and its mitogenic activity for endothelial cells. Although the role of VEGFR-1 is unclear in adult angiogenesis, VEGFR-2 is essential for VEGF biological responses (Ferrara, 2001).

Another existing receptor, VEGFR-3 (Flt-4), appears to be primarily involved in lymphatic angiogenesis. It binds to VEGF-C and –D but not to VEGF-A. PIGF and VEGF-B only bind to VEGFR-1 and appear to have little angiogenic activity. VEGF-E appears to be a selective ligand of VEGFR-2 and strongly induces angiogenesis.

VEGF-A, as mentioned before, binds to VEGFR-1 and –2 but not to VEGFR-3 (Ferrara, 2001).

1.7.4 Role of VEGF in reproduction

1.7.4.1 Role of VEGF during follicular development

Follicular and CL growth and development, as well as endocrine function, are dependent on blood vessel proliferation. It has been suggested that VEGF may act as mediator of blood vessel growth that occurs in the female reproductive tract (Ferrara et al., 2003a).

VEGF is essential for follicular development, as suppression of VEGF inhibits the development of pre-ovulatory follicles. The lack of VEGF is also known to cause a decrease in theca layer vascularity, granulosa cell proliferation, antral formation and steroidogenesis. Absence of VEGF disrupts ovulation and subsequent CL development and function (Hunter et al., 2004, Ferrara et al., 2003a).

In response to FSH, granulosa cells are the main source of VEGF in the follicle and its expression increases as follicles mature, but theca cells are also able to express VEGF. The VEGF produced by the granulosa cells accumulate in the follicular fluid and this is believed to be the source of stimuli for blood vessel extension towards the granulosa layer around the time of ovulation in cattle, primates and pigs (Robinson et al., 2007, Hunter et al., 2004, Mattioli et al., 2001).

VEGF receptors, on the other hand, are found mainly in endothelial cells of the theca layer, suggesting that VEGF acts in a paracrine fashion to stimulate vascularisation of the theca layer (Hunter et al., 2004, Wulff et al., 2001a). Both theca and granulosa cells express VEGF mRNA in healthy porcine follicles; however, the granulosa compartment expresses more intense hybridisation signals (Boonyaprakob et al., 2003, Tamanini and De Ambrogi, 2004).

An active cycle of angiogenesis can be seen during follicular development (Ferrara et al., 1992), and numerous studies have shown that the manipulation of the VEGF system can influence follicular development at all stages (Hunter et al., 2004). In granulosa cells of primordial and pre-antral follicles VEGF mRNA and protein are

very weak or undetectable (Ferrara et al., 2003a, Tamanini and De Ambrogi, 2004). In secondary follicles VEGF protein and mRNA expression is still very weak within the granulosa layer, while intense binding can be observed within vessels of the theca layer (Tamanini and De Ambrogi, 2004, Ferrara et al., 1992, Ferrara et al., 2003a). However, in primates, VEGF mRNA can first be detected in late secondary follicles and appear to be in equal quantities in granulosa and theca layers (Wulff et al., 2001a).

As the follicle matures, VEGF mRNA expression progressively increases and antral follicles show intense granulosa cell signal with moderate VEGF mRNA expression in the theca layers (Ferrara et al., 2003a, Mattioli et al., 2001, Ravindranath et al., 1992, Tamanini and De Ambrogi, 2004, Wulff et al., 2001a). In ovine pre-ovulatory follicles, VEGF mRNA expression is only detectable in the theca interna (Tamanini and De Ambrogi, 2004). This mRNA correlates quite well with the vascular changes taking place within the developing follicle (Ferrara et al., 1992). An increase in both VEGF and VEGFR levels in tertiary follicles is probably responsible for follicular growth and antrum formation (Wulff et al., 2001a) as VEGF permeabilization action is thought to be essential for antrum formation (Tamanini and De Ambrogi, 2004).

VEGF is thought to play an important role in the selection of the dominant follicle as VEGF concentrations in follicular fluid were found to be associated with increased vascular density and oestrogen concentrations (Hunter et al., 2004).

In pig follicles, VEGF production is regulated according to follicular size and functional status. Small follicles (<4 mm) have low levels of VEGF while large follicles (>5 mm) are able to produce substantial amounts of VEGF (Mattioli et al., 2001). The same can be observed with VEGFR-1 and -2 expressions in the theca layer (Tamanini and De Ambrogi, 2004). Medium follicles (4 to 5 mm), however, may or may not produce high amounts of VEGF and are classified as high or low-VEGF producers. The border line for significant VEGF production seems to be 4 to 5 mm since it has been observed that follicles bigger than that produce high levels of VEGF. This suggests that under gonadotrophin stimulation follicles bigger than 4mm become capable of stimulating angiogenesis and probably creating the required conditions for their pre-ovulatory development (Mattioli et al., 2001).

Medium sized porcine follicles show 2 concentric vascular networks that are positively related to VEGF levels found in follicular fluid. Follicles with high levels of VEGF have a total vascular area greater than those with low levels of VEGF. The increase in vascular area involves both the inner (within theca interna by the basal membrane) and outer (within the theca externa) vascular networks. It has also been observed that there is a positive relation between VEGF and oestrogen levels in follicular fluid. High levels of oestrogen observed in follicular fluid when high levels of VEGF are present suggest that VEGF producing follicles are those that will effectively grow and ovulate or will at least reach the pre-ovulatory stage. The positive relation between VEGF and oestrogen levels suggest a possible cause-and-effect relationship, although, it has been shown that oestrogen does not stimulate VEGF production *in vitro* (Mattioli et al., 2001). Activation of steroidogenesis requires an increased supply of nutrients, and VEGF is known to create these conditions and also to increase vascular permeability, facilitating the delivery of lipoproteins for steroid production. Therefore, it is possible that VEGF stimulates oestrogen production by providing steroidogenic cells with the required nutrients (Mattioli et al., 2001).

Many factors are thought to regulate follicular VEGF production. Gonadotrophins stimulate granulosa cell production of VEGF and hypoxia is a potent inducer of VEGF expression in a number of tissues (Hunter et al., 2004). IGF-I acts as stimulant to VEGF as well as directly acting on endothelial cell proliferation and differentiation (Schams and Berisha, 2004).

As follicles mature angiogenesis slows down and blood vessels stabilize. However, it is known that VEGF upregulates vascular permeability, thus during this period VEGF might be present at high levels to enhance vascular permeability rather than to promote angiogenesis (Hayashi et al., 2003). In the peri-ovulatory period, VEGF levels increase markedly in follicular fluid and peptide can be detected in granulosa-derived luteinized cells of the early CL (Tamanini and De Ambrogi, 2004, Ferrara et al., 1992).

Degradation of the capillary bed interrupts metabolite supply and leads to follicular atresia. In follicular atresia in cattle, apoptosis is first observed in the outer theca capillaries. In pigs, however, atresia does not affect other capillary networks

due to basal VEGF production by the theca layer (Tamanini and De Ambrogi, 2004). During follicular atresia in primates, VEGF can be reduced up to 55% in granulosa and is no longer detectable in the theca layer. A decrease in 50 to 70% of VEGFR-1 can be seen in atretic follicles when compared to secondary or tertiary follicles (Wulff et al., 2001a). In humans, VEGF mRNA is only weakly expressed in residual theca cells (Ferrara et al., 2003a).

Blocking of VEGF decreases endothelial and non-endothelial cell proliferation. This effect on non-endothelial cells could be due to the lack of nutrients, hormones and growth factors that reach these cells due to the reduced local permeability caused by the absence of VEGF (Tamanini and De Ambrogi, 2004). In some species such as pigs, hypoxia can induce VEGF production. As the follicle matures, the pressure of oxygen (pO_2) gradually decreases in follicular fluid, acting as an angiogenic stimulus. In cows, *in vitro* exposure to low pO_2 stimulates VEGF mRNA expression. However, in primates, hypoxia does not interfere with VEGF expression in granulosa cells (Tamanini and De Ambrogi, 2004).

In pigs, equine Chorionic Gonadotrophin (eCG) administration increases VEGF mRNA expression in granulosa cells, VEGF levels in follicular fluid and VEGFR-1 and –2 in theca but no difference can be observed in VEGF mRNA expressed in the theca layer. It has been observed that in some species fasting increases VEGF expression in theca cells while reducing it in granulosa cells. LH and IGF-1 stimulate VEGF expression in bovine and primate cultured granulosa cells. Gonadotrophins, in the primate, stimulate VEGF secretion in pre-ovulatory follicles (Tamanini and De Ambrogi, 2004).

Local injection of VEGF gene fragments increases the population of large follicles and the vascular density in the theca interna. Plasma VEGF concentrations are directly proportional to number of oocytes recovered, indicating that enhancing VEGF expression during follicular development can increase the number of follicles destined to ovulate. Gonadotrophin administration could be used to enhance VEGF production and therefore promote the development of a higher number of oocytes. In humans, VEGF concentration in follicular fluid is related to pure FSH administration (Tamanini and De Ambrogi, 2004).

1.7.4.2 Role of VEGF during luteal development

After ovulation, the endothelial vessels invade the ruptured follicle and form a network of microvessels that supply the developing CL (Ferrara et al., 1992). VEGF can be found during all stages of the CL life-span: development, function and regression (Schams and Berisha, 2004).

Suppression of VEGF causes a significant reduction in endothelial cell proliferation, with a 75% reduction in the area occupied by these cells. It also leads to increased cell apoptosis and a 50% reduction in plasma progesterone concentration (Wulff et al., 2001b, Ferrara, 2001, Dickson et al., 2001). The lack of VEGF increases VEGF gene expression, probably as compensation and is likely associated with the stimuli caused by local hypoxia due to lack of vascularisation. Decreased levels in VEGF also result in decreased levels of VEGFR-1 mRNA, indicating that VEGF regulates its own receptor expression (Wulff et al., 2001b).

Although the lack of VEGF has no apparent effect on the pre-existing vasculature (Ferrara, 2001), indicating a certain independence of the other angiogenic factors from VEGF, luteal angiogenesis is primarily driven by VEGF and a proportion of endothelial cells are dependent on VEGF support (Dickson et al., 2001, Wulff et al., 2001b).

VEGF is usually detected in theca derived luteinized cells of early CL. However, in primates all luteal cells express VEGF mRNA (Tamanini and De Ambrogi, 2004). In primates VEGF mRNA is highly expressed in steroidogenic luteal cells while no expression has been observed in endothelial cells. On the other hand, VEGFR-1 was found exclusively in endothelial cells of primates CL (Wulff et al., 2001b).

In pigs CL, $VEGF_{165}$ and both receptors have been detected (Tamanini and De Ambrogi, 2004). VEGF mRNA prevails in large luteal cells (granulosa derived) and pericytes, while VEGFR-1 and -2 mRNA can be found mainly in the theca derived cells (Tamanini and De Ambrogi, 2004, Boonyaprakob et al., 2003). In mares, VEGF mRNA is expressed in luteal cells and absent in endothelial cells. VEGF protein is mainly found in the cytoplasm of luteal cells (Tamanini and De Ambrogi, 2004, Al-zi'abi et al., 2003).

Isoforms 121, 165 and 189 have been identified in both ovine and bovine CL. While bovine CL express predominantly isoforms 121 and 165 (Schams and Berisha, 2004), isoform 121 corresponds to 1/3 of total luteal VEGF found in sheep. Steroidogenic luteal cells are active VEGF producers, alongside pericytes and vascular smooth muscle cells (Tamanini and De Ambrogi, 2004). Vascular smooth muscle cells have been shown to synthesize VEGF mRNA and secrete biologically active VEGF isoforms 121, 165 and 189 (Tischer et al., 1991). Pericytes are the first cells to migrate to the hypoxic granulosa after ovulation, and to produce VEGF to initiate angiogenesis. In bovine CL both VEGFR-1 and -2 mRNA are only expressed by endothelial cells (Tamanini and De Ambrogi, 2004).

VEGF levels are high during early luteal phase and decrease as the oestrous cycle reaches an end (Kaczmareck et al., 2005, Ribeiro et al., 2006). During the mid-luteal phase high levels of VEGF can be seen in luteal cells (Robinson et al., 2007) but tend to be confined to those cells adjacent to the tuberculae (Al-zi'abi et al., 2003). During early and mid luteal phases intense VEGF mRNA expression can be seen and early luteal VEGF mRNA expression resembles the pattern seen in late pre-ovulatory follicles with granulosa derived cells intensely expressing VEGF mRNA. VEGF mRNA expression appears to be stronger during initial recruitment and capillary development during the early luteal phase (Ferrara et al., 2003a) with constant levels throughout the mid-luteal phase and a significant decrease as it reaches an end (Ribeiro et al., 2006, Tamanini and De Ambrogi, 2004). Besides being produced by steroidogenic luteal cells VEGF is also produced by pericytes. Although pericytes are still present at mid-cycle many of them are not producing VEGF. This probably occurs because at this stage the CL has obtained its maximum size, functionality and vascular supply (Redmer et al., 2001).

By early luteal regression, luteal cells express very little VEGF protein and mRNA expression decreases markedly. However, during late regression VEGF protein and mRNA expression are significantly increased in non-luteal cells such as macrophages and neutrophils. The number of immune cells expressing VEGF mRNA increases during regression. It is thought that VEGF at this stage is only required to maintain the remaining microvessels involved in clearing degenerating luteal cells (Al-zi'abi et al., 2003, Tamanini and De Ambrogi, 2004).

VEGFR-2 levels follow a similar pattern to that of VEGF protein (Robinson et al., 2007, Boonyaprakob et al., 2003) but high levels of VEGF and VEGFR-2 are only maintained during the late luteal phase in cases of pregnancy (Tamanini and De Ambrogi, 2004).

The peak expression of VEGF mRNA and protein during the early luteal phase shows a direct association with high endothelial cell proliferation and the establishment of a dense capillary network, indicating a close association with angiogenesis. During the early luteal phase, when new capillaries are being formed throughout the CL, VEGF expression is diffused amongst cells. When this capillary network has been established by the mid-luteal phase, VEGF levels are high only in cells bordering the tuberculae, where blood vessels enter the luteal tissue (Al-zi'abi et al., 2003, Ferrara et al., 1992).

Endometrial PGF-2α-induced luteolysis consists of functional and structural luteolysis (Niswender and Nett, 1994). PGF-2α exerts a significant impact on the VEGF system, significantly reducing mRNA expression of VEGF and its receptor. However, soon after PGF-2α release VEGF mRNA levels rise, probably due to the acute hypoxia created by the death of endothelial cells. By 24 hours after PGF-2α release, a decrease in VEGF mRNA can be observed, and it is hypothesised that this initiates the definite loss of capillary endothelial cells (Vonnahme et al., 2006).

Recently, another endothelial growth factor has been identified, the Endocrine Gland-derived Vascular Endothelial Growth Factor (EG-VEGF). This factor has been shown to be expressed in the primate and human ovaries. EG-VEGF is able to induce a strong angiogenic response in the ovary but fails to do so in other organs such as skeletal muscle. Similar to VEGF, EG-VEGF mRNA expression is up-regulated by hypoxia. EG-VEGF mRNA has been found to be expressed at times when VEGF mRNA expression is weak or absent, during both follicular and luteal phases. EG-VEGF mRNA is expressed in primary follicles when VEGF mRNA is barely detectable (Ferrara et al., 2003a).

EG-VEGF is structurally different from VEGF but has a similar function. VEGF and EG-VEGF mRNA are largely similar in their localization in the CL, predominantly in large luteal cells. However, it has been observed that, in humans, VEGF mRNA total expression does not significantly change during early, mid and

late luteal phase while EG-VEGF progressively increases throughout the luteal phase. EG-VEGF is predominantly localized within large luteal cells, with uniform distribution throughout the CL. During the early luteal phase EG-VEGF mRNA is weakly expressed in granulosa-derived cells and at a lower level in theca-derived ones. By the mid-late luteal phase granulosa-derived cells express intense mRNA expression and theca-derived cells are negative (Fraser et al., 2005).

1.8 The Angiopoietin family

1.8.1 Molecular biology of Angiopoietins

Angiopoietins are angiogenic factors which act in association with VEGF to regulate blood vessel formation and maturation (Davis et al., 1996). In mice three types of angiopoietins have been detected: Angiopoietin (Ang)-1, -2 and -3, while Ang-4 has also been found to be present in humans (Tamanini and De Ambrogi, 2004, Wang et al., 2006).

Ang-1 is a 497-amino acid glycoprotein with a molecular weight of approximately 55 kDa under reducing conditions. Although Ang-1 is not capable of mediating endothelial cell growth of tubule formation, like VEGF, it is nevertheless essential for vascular development (Davis et al., 1996, Suri et al., 1996, Koblizek et al., 1998). The lack of Ang-1 results in less complex vasculature with less distinction between small and large vessels, as well as reduced number of large vessels and defects in vascular remodelling. The absence of Ang-1 causes endothelial cells to poorly associate with the underlying matrix of blood vessels and not properly recruit pericytes and vascular smooth muscle cells. This poor association with the underlying cells leads to the defects in vascular organization observed in animals lacking Ang-1 (Suri et al., 1996).

Ang-1 is believed to regulate vascular maturation after the initial stages of blood vessel formation (Davis et al., 1996), and it has been shown to be essential for embryo survival (Suri et al., 1996). Its expression in the vicinity of developing vessels suggest that Ang-1 plays an important role in assembling non-endothelial vessel wall components such as vascular smooth muscle cells and pericytes, which

will ensure blood vessel stabilization and maturation (Tamanini and De Ambrogi, 2004, Folkman and D'amore, 1996).

Ang-1 binds to the endothelial specific TIE-2 receptor tyrosine kinase and activates the production and release of recruiting signals for mesenchymal cells. The endothelium TGF-β induces differentiation of these mesenchymal cells into pericytes and smooth muscle cells. TIE-2 activation by Ang-1 inhibits endothelial cell proliferation and stimulates matrix deposition (Folkman and D'amore, 1996). Although Ang-1 is mainly involved in vessel maturation, low levels of Ang-1 act synergistically with VEGF to induce the formation of new capillaries by sprouting from pre-existing vessels (Koblizek et al., 1998).

Ang-2 is a 496-amino acid protein of approximately 65 kDa (Geva et al., 2002) that binds to TIE-2 receptors with a similar affinity to Ang-1. Although both factors bind to the same receptor, they have different physiological roles. While Ang-1 causes TIE-2 phosphorylation in endothelial cell lines, Ang-2 has no such activity, even at high levels. However, in non-endothelial cell lines, Ang-2 is capable of inducing TIE-2 phosphorylation as much as Ang-1, suggesting that endothelial cells contain unique components that interact with TIE-2, allowing functional discrimination between the two angiopoietins (Maisonpierre et al., 1997).

Ang-2 destabilizes existing vessels, loosening the supporting cell matrix and allowing VEGF to stimulate endothelial cell proliferation and migration to result in new blood vessel formation (Tamanini and De Ambrogi, 2004).

Ang-2 in high concentration blocks Ang-1 activity in endothelial cells. Over-expression of Ang-2 causes widespread vessel discontinuity and loss of normal dendritic patterns of the vascular network, giving it a “moth appearance”. Characteristic vessels are frequently absent and endothelial cells are present in abnormal small clusters in the vicinity of the vessels which are present. This finding supports the notion that Ang-2 is an antagonist to Ang-1 in endothelial cells (Maisonpierre et al., 1997). As Ang-2 is unable to cause TIE-2 phosphorylation in endothelial cells, it antagonises Ang-1 activity by competing for the same receptor. As the affinities of both ligands to TIE-2 are similar, higher levels of Ang-2 result in less receptors being available for Ang-1 induced phosphorylation.

The Ang-2:Ang-1 ratio is considered as an index of destabilization of blood vessels (Shimizu et al., 2007). However, whether this destabilization will lead to the formation or regression of blood vessels is highly dependent on VEGF levels. When Ang-2:Ang-1 ratio and VEGF levels are high, angiogenesis results in the formation of new blood vessel networks. However when Ang-2:Ang-1 ratio is high but VEGF levels are low, regression of blood vessels can be observed. Furthermore, low Ang-2:Ang-1 ratio and relative low levels of VEGF result in stabilization of blood vessels (Hayashi et al., 2003), as illustrated in Figure 3.

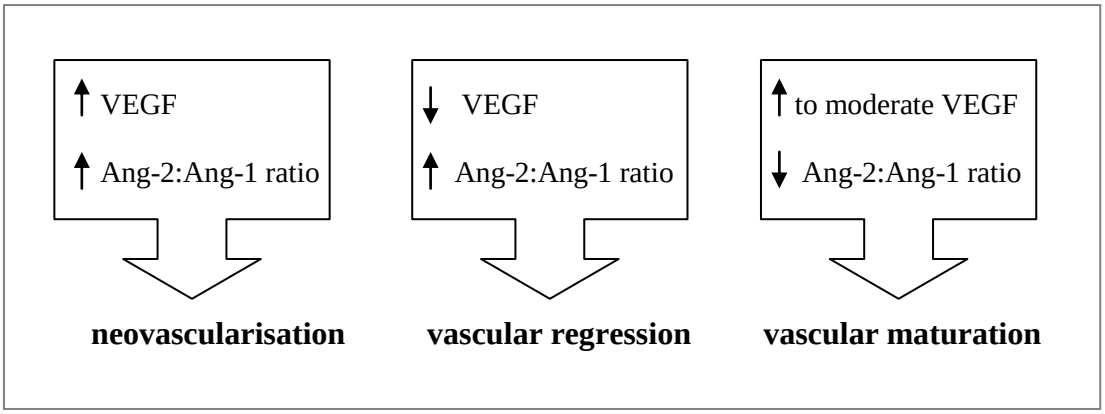


Figure 3. Diagram illustrating the interactions between angiogenic factors and their influence on angiogenesis.

1.8.2 TIE Receptors

The TIE receptor family consists of TIE-1 and TIE-2 (Tek) and is critically involved in angiogenesis. These receptors play a critical vessel remodelling role subsequent to the actions of VEGF (Folkman and D'amore, 1996). These two tyrosine kinase receptors, however, seem to have distinct roles in the formation of blood vessels (Sato et al., 1995).

TIE-2 is an 1122-amino acid glycoprotein of approximately 140-145 kDa, with an extracellular domain and an intracellular tyrosine kinase domain (Vikkula et al., 1996, Runting et al., 1993). The overall amino acid homology between TIE-2 and TIE-1 is approximately 46%. However, this similarity varies in the different domains, reaching as high as 84% in the kinase domain (Iwama et al., 1993).

TIE-2, when phosphorylated, recruits surrounding stromal cells to stabilize vessel structure, and is necessary for capillary fluid exchange (Tamanini and De

Ambrogi, 2004) and is essential for embryonic survival (Suri et al., 1996). Overexpression of TIE-2 receptor results in venous malformations, with extremely distended blood vessels that show abnormal distribution of smooth muscle cells (Vikkula et al., 1996).

Even though TIE-2 binds with the same affinity to Ang-1 and Ang-2, it has been shown that Ang-1 is the primary physiological ligand for this receptor (Suri et al., 1996). Previous studies have suggested that the number of TIE-2 receptors is regulated by the availability of Ang-1 and not the other way around, or that this regulation is performed indirectly as TIE-2 levels could depend on the degree of vascular organization and not on the availability of the ligand (Suri et al., 1996, Folkman and D'amore, 1996).

The TIE-1 receptor, like TIE-2, is expressed predominantly in endothelial cells (Maisonpierre et al., 1997). Animals deficient in TIE-1 fail to establish structural integrity of vascular endothelial cells, and develop oedema and localized haemorrhage. Although involved in different aspects of vascular maturation, TIE-1 is essential for embryo survival (Sato et al., 1995). Curiously, neither Ang-1 nor Ang-2 is known to bind to this receptor (Maisonpierre et al., 1997).

1.8.3 Role of Angiopoietins in reproduction

In adult human tissues, Ang-1 is widely expressed while Ang-2 is restricted to predominant sites of physiological vascular remodelling: ovary, placenta and uterus. It has been observed that in the rat ovary Ang-1 transcripts were associated with blood vessels and coincided with vessel in-growth into the early CL, rather than preceding it as does VEGF. These findings are in agreement with the hypothesis that Ang-1 has a later role in angiogenesis than VEGF, probably involving vessel maturation and stabilization. Ang-2 pattern, on the other hand, suggests that this factor plays an early role at the sites of vessel invasion, collaborating with VEGF by blocking the stabilizing or maturing function of Ang-1 and thus allowing vessels to remain more plastic and better responsive to sprouting signals from VEGF (Tait and Jones, 2004, Maisonpierre et al., 1997).

1.8.3.1 Role of Angiopoietins during follicular development

Ovarian follicle development depends on the balance between vascular outgrowth and regression (Maisonpierre et al., 1997).

The theca interna of mature bovine follicles expresses mRNA encoding Ang-1, Ang-2, TIE-1 and TIE-2 while the granulosa layer only signals for mRNA encoding Ang-1 and Ang-2. Ang-1 mRNA expression does not vary between developing and mature follicles, but on the other hand, Ang-2 mRNA expression is significantly lower in mature follicles than in developing ones (Hayashi et al., 2003, Tamanini and De Ambrogi, 2004, Shimizu et al., 2007). Thus, the Ang-2:Ang-1 ratio is lower in the theca interna layer of mature follicles when compared to that of developing ones. This shows that as follicular maturation progresses, the Ang-2:Ang-1 ratio shifts from high (destabilized vessels) to low (stabilized vessels). A reduction in the Ang-2:Ang-1 ratio in mature follicles indicate blood vessel maturation and reduced angiogenesis despite high levels of VEGF. TIE-1 and TIE-2 mRNA expression remain unchanged throughout follicular development (Hayashi et al., 2003).

In the theca layer of tertiary follicles, Ang-1 mRNA is expressed at low levels and some authors report that Ang-2 mRNA cannot be detected at any stage of follicular development. TIE-2 mRNA, on the other hand, has been detected in as early as primordial follicles. Unlike in other species, TIE mRNA expression in primates is present in the granulosa layer of primary and secondary follicles. These levels, however, decrease by the late secondary stage and are barely detectable in the granulosa of tertiary follicles. TIE-2 mRNA expression can be mainly detected in theca cells of tertiary follicles and decrease by 70% as follicles undergo atresia (Wulff et al., 2001a).

It has been shown that Angiopoietins are associated with the changes in follicular capillaries at the time of the LH surge. Ang-1 mRNA is highly expressed before the LH surge, followed by a significant decrease at the time of the LH surge. A slow and consistent rise is observed after the LH surge, followed by a slow decrease at peri-ovulatory time and early CL development. It is believed that the reduction in Ang-1 mRNA expression in peri-ovulatory follicles may be directly regulated by gonadotrophins. Ang-2 mRNA levels are steady throughout follicular development

and ovulation with lowest levels soon after the LH surge and at peri-ovulatory time (Shimizu et al., 2007).

The Ang-2:Ang-1 ratio is increased at the time of the LH surge. An increase in this ratio is associated with blood vessel destabilization, which is essential for angiogenesis. Therefore, it is likely that during the LH surge the follicle prepares the capillary network for ovulation. The Ang-2:Ang-1 ratio gradually declines by ovulation time and is significantly increased in early CL development. TIE-2 mRNA is found at its highest levels prior to the LH surge and at early stages of CL development, while lower levels are observed at peri-ovulatory time (Shimizu et al., 2007).

Ang-1 is associated with an increase in oestrogen secretion by the dominant follicle as well as in stimulating, in synergism with Ang-2, early luteinisation and progesterone release from peri-ovulatory follicles after the LH surge (Hayashi et al., 2003).

During follicular atresia, when large follicles fail to ovulate, there is no invasion of vessels into the granulosa and those vessels present in the theca interna recede as the follicle regresses. VEGF mRNA is virtually undetectable in atretic follicles whereas Ang-2 mRNA is present in large amounts within the granulosa (Maisonpierre et al., 1997). In the theca layer, Ang-1 and Ang-2 mRNA expression do not change throughout follicular atresia but Ang-2 expression seems to be always higher than Ang-1. High Ang-2:Ang-1 ratio and low VEGF turns blood vessels towards regression in atretic follicles (Maisonpierre et al., 1997, Hayashi et al., 2003, Tamanini and De Ambrogi, 2004).

TIE-1 and TIE-2 mRNA expression are also higher in early atretic follicles but TIE-2 mRNA levels decrease markedly by the mid and late stages of atresia (Tamanini and De Ambrogi, 2004, Hayashi et al., 2003).

1.8.3.2 Role of Angiopoietins during luteal development

The VEGF and Angiopoietin-TIE signalling systems are essential for follicular and luteal angiogenesis (Shimizu et al., 2007). Ang-1 levels are mainly associated with vascular invasion in the formation of the new CL while Ang-2 expression

suggests that it plays an earlier role than Ang-1 at the sites of vessel formation. Ang-2 clusters are abundant in the front of the vessels invading the newly formed CL (Maisonpierre et al., 1997).

During early CL development high Ang-2 and low Ang-1 expression result in a high Ang-2:Ang-1 ratio. This ratio is mainly dictated by Ang-1 level variation as Ang-2 levels are thought to remain stable throughout CL development. Ang-1 levels increase during the mid luteal phase resulting in a low Ang-2:Ang-1 ratio followed by higher Ang-2:Ang-1 ratio due to decreasing Ang-1 levels during the late luteal phase (Schams and Berisha, 2004, Sugino et al., 2005). The different levels of VEGF will determine if unstable blood vessels are to result in angiogenesis or vascular regression (Schams and Berisha, 2004).

The variations of these angiogenic factors and the vascular network present during the different luteal development stages clearly demonstrate the close influence of these factors in the formation, stabilization and regression of the luteal vascular network (Sugino et al., 2005).

Degenerating CLs show similar patterns of VEGF and Angiopoietins as atretic follicles. PGF-2α shows no effect on Ang-1 mRNA expression levels but increases Ang-2 mRNA when VEGF mRNA starts to decline (Vonnahme et al., 2006). Therefore degenerating CLs show small amounts of Ang-1 and VEGF mRNA expression while Ang-2 mRNA is expressed in large amounts (Maisonpierre et al., 1997).

Although blood flow has been long recognized as an important factor in ovarian function, recent data indicate that angiogenic factors and their receptors may play a major role in regulating follicular development and maintenance of progesterone levels compatible with pregnancy (Hunter et al., 2004).

1.9 Hypothesis and objective of the study

It has been repeatedly proven that small-dose multiple injections of GnRH are capable of consistently inducing ovulation in seasonally anoestrous ewes (McLeod et al., 1982b, McLeod et al., 1982a, McLeod et al., 1983, McNatty et al., 1982, Southee et al., 1988b, Southee et al., 1988a), and that a defective CL is formed as a result in the majority (approximately 75%) of animals (Southee et al., 1988a, Southee et al., 1988b, McLeod et al., 1982b). Defective luteal function, however, is not only observed in seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH but is also a common phenomenon after the first ovulation at puberty, or following parturition over a range of species and is therefore considered to affect the fertility adversely.

These observations provide a controlled *in vivo* model of defective luteal function caused by GnRH-induced ovulation in the seasonally anoestrous ewe. This enables what is normally observed after the first ovulation at puberty, or following parturition or seasonal anoestrus to be reproduced.

It has also been extensively shown that progesterone priming of seasonally anoestrous ewes for as early as 3-5 days prior to GnRH treatment results in 100% normal luteal function against only 25% of non-primed animals (McLeod et al., 1982b, Keisler and Buckrell, 1997, Hunter et al., 1988, Husein and Kriddli, 2003, Haresign et al., 1996, Hunter et al., 1989, Southee et al., 1988b, Southee et al., 1988a, Bartlewski et al., 2001, Pearce et al., 1987). However, it is not known how progesterone priming eliminates defective luteal function. Previous studies, as cited in this literature review, excludes the effect of progesterone priming through the gonadotrophins release from the pituitary gland and/or PGF2-α release from the endometrium. Collectively, it seems that progesterone given prior to induction of pre-ovulatory follicle maturation can affect what happens after ovulation.

It is also well documented that an adequate vascular bed is essential for normal follicular and luteal development and that angiogenic factors such as VEGF and Angiopoietins and their receptors are essential for blood vessel formation and maturation (Maisonpierre et al., 1997, Shimizu et al., 2007, Berisha and Schams, 2005, Kaczmareck et al., 2005, Reynolds et al., 2000, Redmer et al., 2001, Miyamoto

et al., 2005, Nett et al., 1976). A question arises: is there any possibility that progesterone priming can eliminate the defective luteal function by affecting the vascular development either in the pre-ovulatory follicle and/or early *corpus luteum*? Indeed there is some, although limited, information to support this as progesterone has been shown to stimulate the expression of VEGF and VEGFR-2 in the bovine granulosa cells when cultured *in vitro* (Shimizu and Miyamoto, 2007). It is, therefore, hypothesized that progesterone priming of seasonally anoestrous ewes, administered with small dose multiple injections of GnRH eliminates defective luteal function by altering the angiogenic system of the selected follicle and early CL.

In order to test this hypothesis both protein and mRNA expression of VEGF, Angiopoietins and their receptors were measured in the follicular layers of pre-ovulatory follicles before and after the LH surge and in the luteal cells of 1, 2 and 4 day old CL of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, with or without progesterone priming.

CHAPTER 2

General Materials and Methods

2.1 Experimental Animals, progesterone priming and GnRH treatment

Fifty seasonally anoestrous Welsh Mule ewes were divided into 10 groups (1 to 10). Each ewe had its jugular vein cannulated to facilitate pulsatile GnRH (Luteinizing Hormone Releasing Hormone human acetate salt, Sigma-Aldrich, L-7134, Lot 053K1109) injections and blood sampling.

Animals from Groups 2, 4, 6, 8 and 10 received a single dose of 20 mg of progesterone (Sigma-Aldrich) in corn oil (i.m.) three days before the start of GnRH treatment while animals in Groups 1, 3, 5, 7 and 9 received oil vehicle alone.

Each animal received injections of 500 ng GnRH (diluted in 2 ml of normal saline solution) given intravenously every 2 hours for 28 hours to induce LH pulses, followed by a bolus injection of 300 μg GnRH at 30 hours after the start of GnRH treatment. The bolus injection was designed to synchronize the onset of the pre-ovulatory LH surge at a time when it is shown it to occur in similarly treated animals (Khalid et al., 1997). This allowed collection of the ovaries at precise times in relation to the LH surge.

Animals from Groups 1 (n = 5) and 2 (n = 5) were slaughtered 24 hours after the start of the pulsatile GnRH treatment (6 hours before the expected LH surge) so that pre-ovulatory follicles prior to the LH surge could be analysed. These animals did not receive GnRH bolus injection.

Animals from Group 3 (n = 5) and Group 4 (n = 5) were slaughtered 46 hours after the beginning of the GnRH treatment (16 hours after GnRH bolus injection) so that pre-ovulatory follicles after the LH surge but prior to ovulation could be analyzed.

Animals from Group 5 (n = 5) and Group 6 (n = 5) were slaughtered 80 hours after the beginning of the GnRH treatment (50 hours after GnRH bolus injection) so that newly formed CL could be analysed (1 day old CL).

Animals from Group 7 (n = 5) and Group 8 (n = 5) were slaughtered 104 hours after the beginning of the GnRH treatment (74 hours after GnRH bolus injection) so that 2 day old CL could be analyzed.

Animals from Group 9 (n = 5) and Group 10 (n = 5) were slaughtered 152 hours after the beginning of the GnRH treatment (122 hours after GnRH bolus injection, 4

days old CL) around the time of regression of the defective CL in animals that were not primed with progesterone (Hunter et al., 1988, Hunter et al., 1989).

The time of the start of pulsatile GnRH treatment and the slaughter times of the different treatment groups of seasonally anoestrous ewes primed or non-primed with progesterone are illustrated in Figure 4.

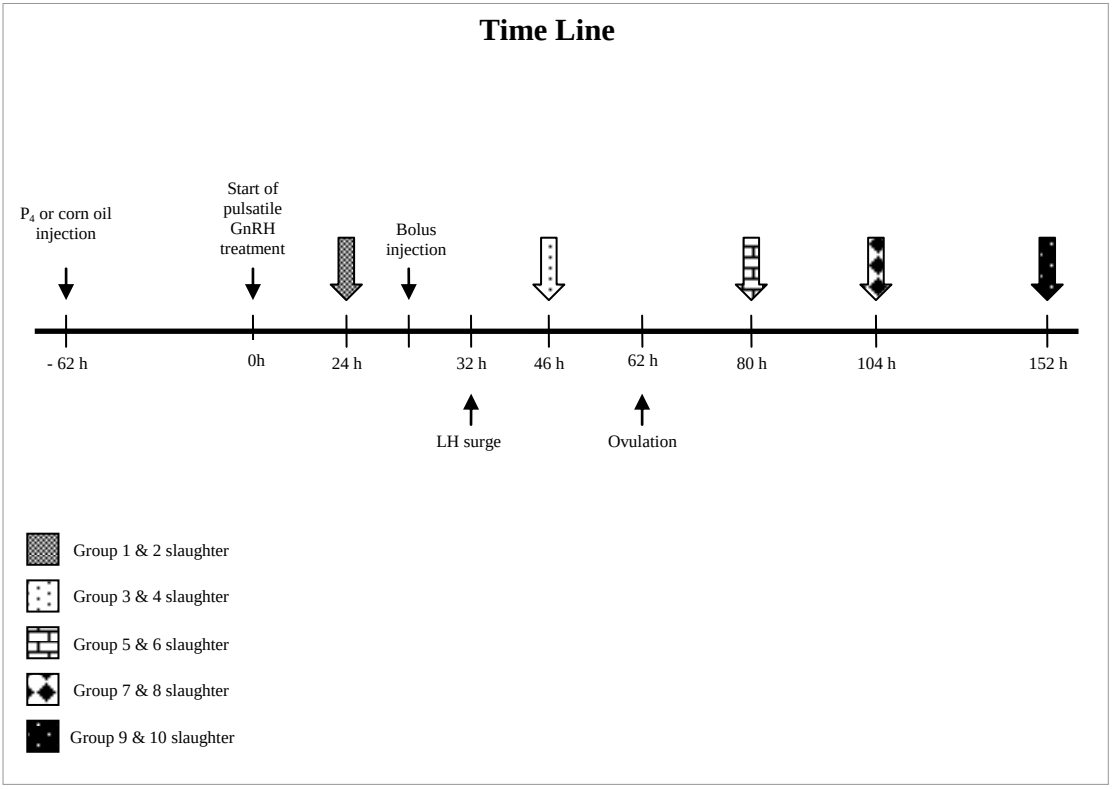


Figure 4. Diagram illustrating slaughter times of Groups 1 to 10 of progesterone primed and non-primed seasonally anoestrous ewes based on the start of pulsatile GnRH injections.

To collect the ovaries animals were killed with an overdose injection of Pentobarbital Sodium (Euthatal®, Merial Animal Health Ltd) in accordance to the Code of Practice for the Humane Killing of Animals under Schedule 1 of the Animals (Scientific Procedures) Act 1986.

The reproductive soundness of the animals used was confirmed by the fact that all ewes had lambed earlier in the year (April). The breeding season for Welsh Mountain ewes start in mid October (Webster and Haresign, 1983), therefore, by performing this experiment in late August it was almost certain that animals were in

seasonal anoestrus with no possible negative interference of prolactin on gonadotrophin stimulation (Webster and Haresign, 1983) as weaning of lambs took place in late June.

2.2 Blood sampling for LH quantification and ovulation monitoring

Animals were blood sampled (3 ml) every 2 hours, just before each GnRH injection, from the start of GnRH treatment until slaughter (Groups 1 and 2) or until 16 hours after GnRH bolus injection (Groups 3 and 4) to monitor the timing of the pre-ovulatory LH peak. Additional samples from ewes in Groups 1 to 4 were taken at 30 minute intervals starting at 6 hours after the start of GnRH treatment for a period of 4h to confirm that injections induced LH pulses (Figure 5).

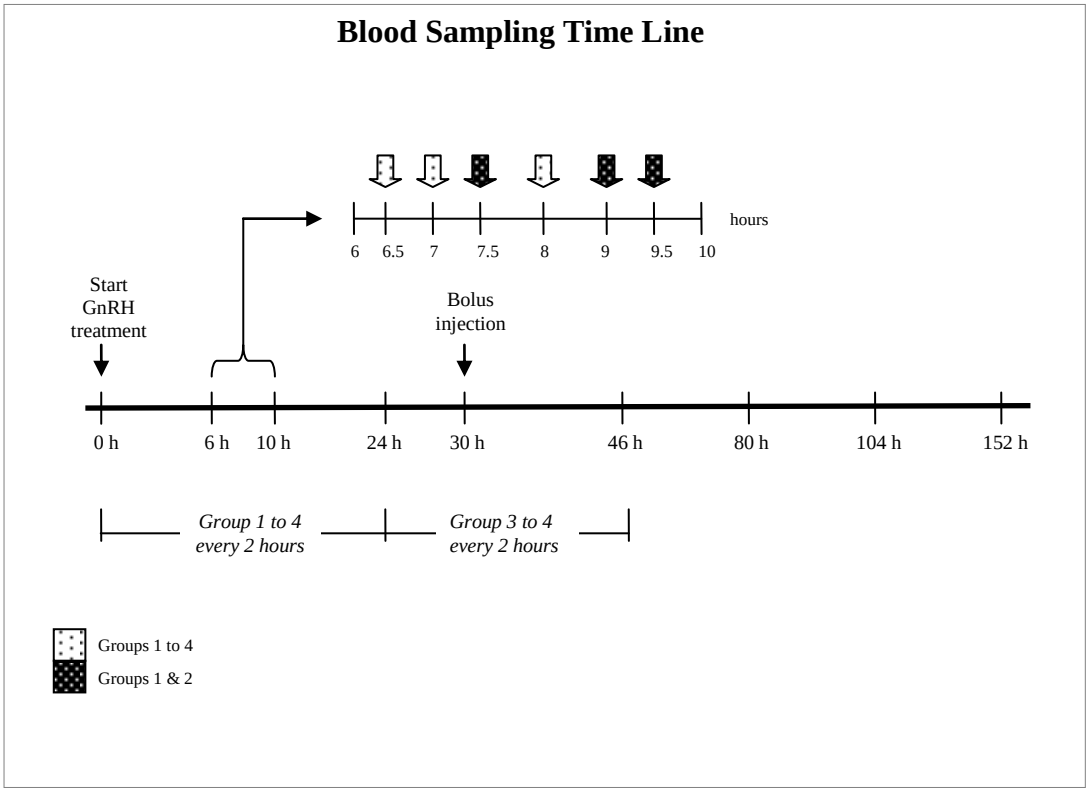


Figure 5. Blood sampling timeline diagram of Groups 1 to 4 of progesterone primed and non-primed seasonally anoestrous ewes based on the start of pulsatile GnRH treatment.

Blood samples were collected into heparinised tubes (L.I.P. Ltd) and centrifuged at 2,500 rpm for 10 minutes at 4 °C. Plasma was carefully transferred into labelled tubes (VWR International) and kept at -20 °C until analysed.

2.3 Tissue Collection

The ovaries from all the animals were collected immediately after euthanasia and washed in 1x Phosphate Buffered Saline (PBS, BDH). Ovaries containing follicles only (Groups 1 to 4) were fixed in 40% v/v neutral buffered formalin for *in situ* hybridization (ISH) and immunohistochemistry (ICH) analysis. If both ovaries had CL, one CL was dissected (for Western Blotting analysis) and rest of the ovary along with the other CL were fixed in 40% v/v neutral buffered formalin (National Diagnostics) for ISH and ICH analysis. If a CL was present in only one ovary, the ovary was sliced in half through the CL and one half of the CL was dissected and used for Western Blotting and the other half along with remaining ovarian tissue was fixed in neutral buffered Formalin for ISH and ICH. Dissected CLs were stored at -80 °C for Western Blotting analysis. For ISH and ICH analysis the ovaries were fixed in formalin for 24 hours then transferred to 70% v/v ethanol (BDH) until embedded in paraffin wax.

2.4 Western Blotting Technique

Western Blotting analysis was done to determine the presence and relative quantity of VEGF, VEGFR-2 (Flk-1), Angiopoietin-1 and -2 and TIE-2 receptor in early corpora lutea (1, 2 and 4 days old). The indirect Western Blotting technique consists mainly of 5 steps: separation of proteins by electrophoresis, transfer of proteins to a membrane, primary antibody probing, enzyme-labeled secondary antibody probing and detection of protein by enzyme degradation of added substrate.

2.4.1 Sample preparation and protein estimation

Frozen corpora lutea samples were taken from the -80 °C freezer and placed on dry ice. The frozen tissues were ground and the powdered CL was placed in a 2 ml microtube. Approximately 5 µg of CL was processed with 300 µl of lysis buffer [0.7 mM Tris (Bio-Rad), 0.02% Sodium Dodecyl Sulphate (SDS, Sigma-Aldrich), 2mM Sodium Orthovandate (Sigma-Aldrich), 1% v/v Glycerol (BDH), 1% v/v Protease Inhibitor Cocktail Set I (Calbiochem) and 4% v/v distilled water]. Samples were homogenized and vortexed until no large pieces of tissue remained. Samples were then spun at 13,000 rpm for 5 minutes at 4 °C. The supernatant was transferred to a clean microtube and heated in a water bath for 5 minutes at 100 ^{o}C and vortexed prior to protein estimation.

Detergent cell lysis is often used in conjuction with tissue homogenization to disrupt cell membranes. Detergents such as SDS break lipid:lipid, protein:protein and lipid:protein interactions disrupting the lipid barrier surrounding cells and solubilising membrane proteins (Pierce Perbio Science Applications Handbook and Catalog 2005/2006). By including SDS in the lysis buffer preparation, both cytoplasmatic (VEGF, Ang-1 and Ang-2) and transmembrane (VEGFR-2 and TIE-2) proteins were freed and able to be analysed.

A 2 µl aliquot of sample lysate was used to determine protein concentration by spectophotometry (NanoDrop® ND-1000 Spectophotometer, NanoDrop Technologies Inc., Wilmington, Delaware, USA). Once the total protein concentration of the sample had been determined, dilution with Lysis Buffer was carried out to achieve a final concentration of 2 µg protein/µl of solution. To each diluted sample 2% bromophenol blue (Sigma-Aldrich) and β-mercaptoethanol (Sigma-Aldrich) were added at a final concentration of 2% and 5%, respectively.

In order to achieve satisfactory protein detection levels it is important to observe that samples must go through purification steps such as denaturation and reduction, amongst others, which aim to make the target protein more accessible to the antibodies being employed. The denaturating temperature for most existing proteins is below 100 °C, therefore submitting the samples to a temperature of 100 °C should cause the rupture of non-covalent bonds of the proteins resulting in a distended

polypeptic chain. The SDS used in the lysis buffer aids the denaturation of the proteins by breaking the hydrophobic interactions within the molecules and thus destroying the protein's native form (Marzzoco and Torres, 1999). β-mercaptoethanol added in excess will denature the protein by reducing disulphide bonds thus overcoming some tertiary protein folding and breaking up quaternary protein structure (Sigma-Aldrich, 2-Mercaptoethanol Product Information Sheet). Samples were kept at -20 ^{o}C until needed for gel electrophoresis.

2.4.2 Gel electrophoresis

The principle of electrophoresis is based on the fact that under the same pH level different proteins have different charges, which will make them migrate at different speeds when submitted to an electric field. Agarose and acrylamide gels enable proteins to be separated by their molecular weight as well as by their charge (Marzzoco and Torres, 1999, Scientific, 2009). Acrylamide is the substance of choice for the preparation of gels and, when in contact with ammonium persulphate, forms a cross-linked polymer network that works as a sieve separating the molecules. The size of the pores formed in a gel is inversely related to the amount of acrylamide used. Therefore, high percentage gels have smaller pores than low percentage ones and are thus better suited for separating small proteins (Scientific, 2009).

Polyacrylamide gel electrophoresis (PAGE) is commonly used to characterize proteins in a specific sample. There are different types of PAGE but the most widely used is the SDS-PAGE technique which separates proteins mainly according to their mass. On the SDS-PAGE system the detergent SDS binds to the hydrophobic radicals of the protein in a pattern of one SDS molecule for every two amino acid residues. The SDS molecules mask the original charge of the native protein by providing a negative charge. This results in a long amino acid complex with negative charge density that is proportional to the length of the polypeptic chain, no matter the protein. This method therefore allows proteins to be separated exclusively by their molecular weight (Marzzoco and Torres, 1999, Scientific, 2009)

Mini one dimension (1D) gels were used for the Western Blotting assays and each gel comprised mainly of two parts: the resolving and the stacking gel. The

resolving gel varied in percentage concentration according to the protein being analysed. High percentage gels were used for smaller proteins and lower percentage gels for heavier proteins, as described in Chapter 4. The resolving gels were composed of a variable concentration of 30% Acrylamide (Protogel®, National Diagnostics), distilled water, Tris (Bio-Rad), SDS (BDH), ammonium persulfate (Sigma-Aldrich) and TEMED (1,2-bis(dimethylamino)-ethane, Sigma-Aldrich).

The composition of resolving and stacking gels used in this project can be found in Table 2. The resolving gel buffer consisted of 1.5M Tris (electrophoresis graded) in distilled water whilst the stacking gel buffer consisted of 0.5M Tris in distilled water, pH adjusted with HCl (BDH Analar®) to 8.4 and 6.8, respectively. The resolving gel was left to set for at least 15 minutes before the stacking gel was added. Stacking gel at 4% was used to create wells into which the samples were loaded. Combs 1.5 mm thick were used to shape wells and the stacking gel was left to set for at least 30 minutes.

Table 2. Resolving and Stacking mini gel formulation (10 ml) for Western Blotting assays.

Gel Percentage	Distilled Water (ml)	30% Acrylamide (ml)	Gel Buffer (ml)	10% w/v SDS (ml)
4%	6.1	1.3	2.5	0.1
6%	5.4	2.0	2.5	0.1
8%	4.7	2.7	2.5	0.1
10%	4.1	3.3	2.5	0.1
12%	3.4	4.0	2.5	0.1
14%	2.7	4.7	2.5	0.1
16%	2.1	5.3	2.5	0.1
For resolving gel add 50 µl of 10% Amonium persulfate and 5 µl TEMED For stacking gel add 50 µl of 10% Amonium persulfate and 10 µl TEMED				

Bio-Rad Mini-Protean 3 Cell Instruction Manual

Equal amounts of extracted protein were loaded into the acrylamide mini gels (8 x 10 cm) and separated by SDS-PAGE. The total amount of protein added varied according to the protein of interest and is further detailed in Chapter 4. For each electrophoresis a negative control (72.5% v/v distilled water, 0.5M Tris-HCl at pH 6.8, 10% v/v glycerol, 5% v/v β-mercaptoethanol, 0.1% w/v bromophenol blue and 2% w/v SDS), a prestained molecular ladder (See Blue Plus2® Prestained Standard,

Invitrogen) and a positive control (an experimental sample used repeatedly) were also loaded into each gel.

Electrophoresis was performed using 1x Tris/Glycine/SDS tank buffer (National Diagnostics) at 150v and 400mA for 60-100 minutes or until samples ran through. Under the effect of an electric current the proteins migrate down the gel matrix creating lanes of protein bands at different molecular weights.

2.4.3 Western Blotting assay

2.4.3.1 Immunoblotting

Once the electrophoresis was done the stacking gel was cut off and the remaining gel containing the proteins was soaked in Transfer Buffer (39mM glycine, 48mM Tris, 0.037% w/v SDS and 20% v/v methanol in distilled water) for 15 minutes. One cut to size polyvinyl difluoride (PVDF) membrane (Immobilon-P® transfer membranes, pore size 0.45μm, Millipore Corporation) per gel was soaked in 100% methanol (BDH) for 5 minutes then washed in water for a further 5 minutes and left in contact with Transfer Buffer for 15 minutes prior to the blotting process. PVDF membranes are hydrophobic in nature and proteins bound to this type of membrane can be used in most immunodetection protocols and can also be detected by most dyes (Hames, 1998).

Thick filter paper (Bio-Rad) was cut to size and soaked in Transfer Buffer alongside the mini gels. A filter paper – membrane – gel - filter paper sandwich was placed in a Semi-Dry Blotting machine (Trans-Blot® semi-dry transfer cell, Bio-Rad Laboratories) and electrophoretic elution was performed at 25v and 33mA/gel (0.8mA per cm^2 gel) for 45 to 90 minutes. Semi-dry blotting is considered to be a quick and complete transfer method (Harlow and Lane, 1988).

2.4.3.2 Blocking and Comassie Blue staining

Transfer of protein was quickly verified by the presence of the prestained markers on the membrane. Thin pieces of sticky fluorescent tape (Glowtape™

luminescent darkroom labeling tape, Sigma-Aldrich) were placed where the molecular weight markers were to facilitate visualization at the end of the process when films were to be revealed. To better check that proteins were satisfactorily transferred, some membranes were stained with Ponceau S (Sigma-Aldrich). The stain reversely binds to the proteins in the membrane and the presence of bands show a successful transfer. Ponceau S can be quickly washed away with distilled water without significant interference with membrane quality.

Membranes were then placed in blocking solution of 10% milk or 5 to 10% Bovine Serum Albumin (BSA, Sigma-Aldrich) in TBST [50mM Tris, 150mM Sodium Chloride (NaCl, Sigma-Aldrich), 0.02% v/v Tween 20 (BDH), dissolved in water and pH adjusted to 7.4] . Blocking was performed for 2 hours or overnight, in a shaker, depending on the protein of interest. The blocking step is essential to minimize background staining. The milk proteins or BSA saturate the binding sites (pores) of the membrane that do not contain the proteins from the gel, thus preventing primary and secondary antibodies becoming trapped in the pores and sending non-specific signals when the detection step is performed (Hames, 1998).

One other way of checking the transfer rate of the proteins was to stain the gels with Comassie Blue solution [0.25% w/v of Comassie Brilliant Blue (Sigma-Aldrich), 2% v/v Methanol (BDH) and 1% v/v Acetic Acid (BDH)] for 1 hour after blotting was over. Comassie blue is an organic dye and consists of one of the simplest methods of protein detection. It is believed that a strong bond is formed between the dye and the protein molecules, especially basic amino acid residues in the proteins (Hames, 1998). After 1 hour the excess dye was washed off by placing gels in a de-staining solution of 10% v/v acetic acid (BDH) and 10% v/v methanol (BDH) in water overnight with an absorbent paper. Blue bands could clearly be seen where there were proteins in the gel (Figure 6).

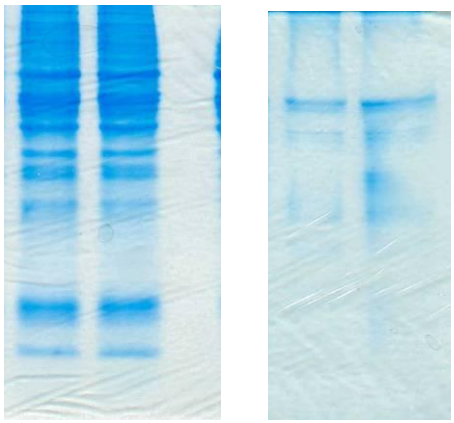


Figure 6. Comassie Blue stained gels before (left) and after (right) semi-dry blotting for 90 minutes. Even though very small amounts of protein can still be seen in the gel after the blotting, the vast majority of the proteins was successfully transferred to the membrane.

2.4.3.3 Primary antibody probing

After blocking, membranes were incubated with 5 ml/membrane of primary antibody solution in a sealed bag at high agitation. Polyclonal antibodies specific to the proteins of interest were used at 1:150 to 1:500 concentration in 5% w/v milk in TBST. Membranes were then washed 4 times in 1x TBST for 15 minutes to remove excess primary antibody. Optimal concentration and incubation period with primary antibody for each protein of interest can be found detailed in Chapter 4.

This project used indirect immunodetection of proteins in which a primary antibody is directed specifically against the protein of interest and is then detected using a secondary labelled antibody directed against the primary antibody (Hames, 1998). This enhances the signal intensity during the detection step, providing better results than the direct method.

2.4.3.4 Secondary antibody probing

Washing was followed by probing with a Horseradish Peroxidase (HRP) labelled secondary antibody, specific to the primary antibody used, for 1 hour at slow agitation. Further details on the secondary antibody used for each protein assay can be found in Chapter 4. Membranes were then washed 4 times in TBST for 15

minutes each time, prior to detection, to remove excess secondary antibody and thus avoid non-specific background staining.

2.4.3.5 Enhanced chemiluminescence detection method

Membranes were placed in Saran wrap and 4 ml of 1:1 ECL solution n°1 and n°2 (ECL™ Western Blot detection reagents, GE Healthcare) was carefully poured onto each membrane and left to incubate for 1 minute. The excess ECL reagent was drained and membranes wrapped in clean Saran wrap and placed in a film cassette. In a dark room, membranes were put in contact with a film sheet (Hyperfilm®, Amersham Biosciences).

Enhanced chemiluminescence is based on the emission of light during the horseradish catalyzed oxidation of luminol. The horseradish peroxidase present in the secondary-primary antibody-protein complex oxidises the substrate quantitatively and the light emitted is captured on film (Figure 7).

The films were exposed to the membranes for 1 to 10 minutes depending on the protein being detected. Further information can be found in Chapter 4. Films were removed from the cassette, placed in developer for 1-2 minutes, rinsed in distilled water and placed in fixer (GBX developer and fixer, Kodak Processing Chemicals for Autoradiography films) for approximately 1 minute. Excess fixer was removed by placing the film in water and films were then hung to dry at room temperature.

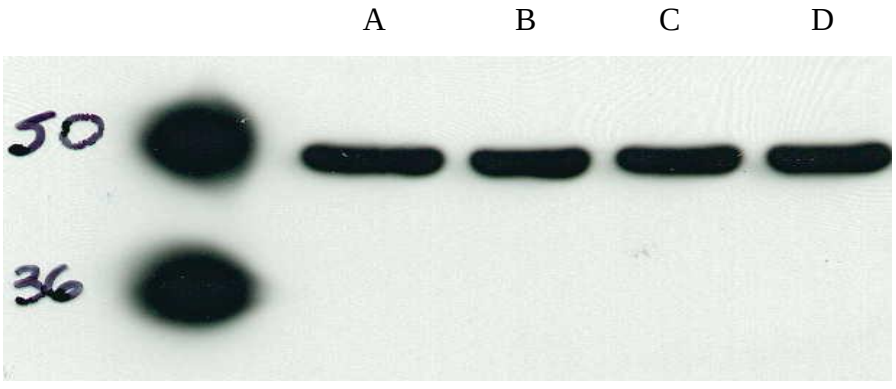


Figure 7. Autoradiograph image of the β-actin band from CL lysate samples (A, B, C and D) at a molecular weight of approximately 50 kDa. Molecular weight markers of 36 and 50 kDa are shown on the left.

2.4.4 Western Blotting analysis

Semi-quantitative analysis of protein content was done by optical densitometry through computer software Quantity One® (Bio-Rad Laboratories). For each sample an area of analysis was drawn around the band and on an area of the background, including the control. The value obtained for each band had the background of its lane subtracted so that any background interference was removed from the analysis. The obtained values were divided by the value of the control and were then compared based on how much of the control they corresponded to. The control sample remained the same for each and every gel, at the same concentration.

2.5 ELISA Immunoassay

Plasma LH quantification was done using a sandwich type Enzyme Linked Immuno Sorbent Assay (ELISA, LH DETECT®, INRA Centre de Tours-PRC, Nouzilly, France) to monitor time of pre-ovulatory LH peak and to monitor LH pulses.

ELISA immunoassays are considered quick and reliable and can be used to determine relative or absolute levels of most protein antigens (Harlow and Lane, 1988). In this ELISA type sandwich assay two polyclonal antibodies produced against the same antigen (LH) were used. The microplate used was sensitized with a purified anti-LH antibody (AC1) prior to use. The LH from the experimental samples binds to the AC1 and is later bound by a second anti-LH antibody (AC2). Reaction intensity is amplified by a peroxidase conjugated antibody (AC3) which binds to the immunocomplex formed. Enzymatic activity is measured using a chromogenic substrate and the reaction intensity is measured by spectophotometry. Reaction intensity is proportional to LH concentration in sample (LH DETECT® instruction manual).

The major advantages of this assay are its specificity and the speed in which it can be performed, as well as the fact that the antigen does not need to be purified. (Harlow and Lane, 1988)

Microplates were removed from the -20 °C freezer and allowed to reach room temperature for about 30 minutes. Meanwhile experimental plasma samples were diluted 1:5 with dilution buffer (provided in the kit) and the standards, positive control and antibody solutions left to thaw at room temperature.

Once at room temperature standards (0, 0.06, 0.12, 0.25, 0.5, 1.0, 2.0 and 3.0 ng/ml) were loaded in duplicate into the first two rows of the microplate. One hundred µl of diluted samples and positive controls were loaded in duplicate to the remaining wells. The plate was covered with adhesive strip and left to incubate for 1 hour at room temperature.

The plates were emptied by inverting onto an absorbent paper and washed 5 times with 0.1% v/v Tween 20 (BDH) in PBS (BDH). Plates were turned and tapped on absorbent paper between washes. To each well 100 µl of anti-LH antibody (AC2) was added and left to incubate for 1 hour at 37 °C. Plates were emptied and washed as described above. After that, peroxidase-conjugated antibody (AC3) was added (100 µl/well) and left to incubate at 37 °C for 1 hour, followed by washing. One hundred µl of substrate solution (TMB) was added to each well and blue colour was left to develop for 10 minutes. One hundred µl of stopping solution was added turning the solutions from blue to yellow (Figure 8) so that measurements using the spectrophotometer could be performed at wavelength of 450nm within 30 minutes of the stopping solution being added.

The concentrations obtained for the experimental samples were based on the comparison with those obtained from the standards and the concentration adjusted by the dilution factor.

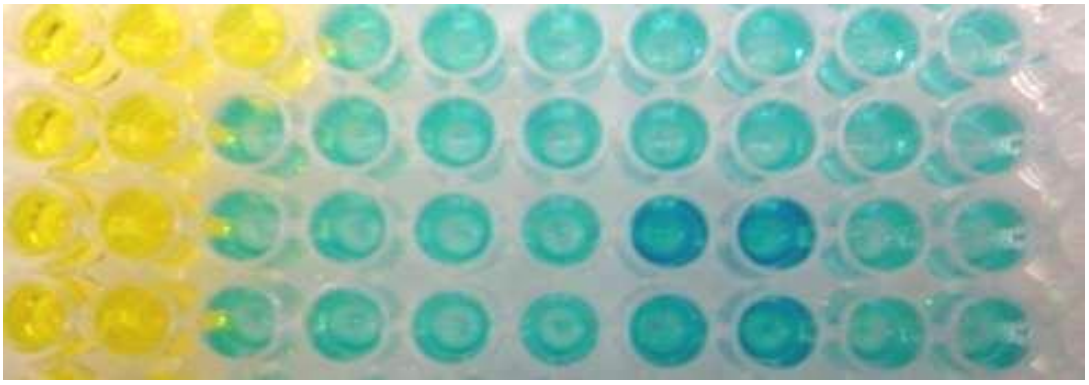


Figure 8. Illustrative LH ELISA Immunoassay microplate following the addition of stopping solution (yellow) to the colour developing reaction (blue).

2.6 Immunohistochemistry

2.6.1 Tissue preparation

2.6.1.1 Fixation

The process of fixation is essential to preserve cells and tissues in a reproducible and life like manner. In addition to altering the chemical nature of cells, fixatives can also cause variable degree of porosity in the tissue allowing penetration of various sized molecules. Non-coagulative fixatives such as formalin result in smaller pore sizes than the coagulant ones. Most fixative solutions are designed to stabilize proteins thus best preserving cellular structure.

Formalin based fixatives are usually well tolerated by cells and have a good tissue penetration which is greatly related to tissue preservation. Some shrinkage and distortion may occur, as can be observed in some follicles, especially those placed close to the ovary border. Formaldehyde fixes by reacting primarily with amino acids to form cross-linking methylene bridges. This results in low permeability to macromolecules and minimal alteration in intracytoplasmatic protein structure. Since the formalin-fixing paraffin-embedding technique has been proven to be superior for preserving cell and tissue morphology, it is one of the most commonly used methods of tissue preservation and preparation for research analysis (Boenisch et al., 2001). Formalin has been the fixer of choice for many ISH and ICH studies using human (Fraser et al., 2005, Ferrara et al., 2003a), equine (Al-zi'abi et al., 2003) and ovine ovaries (Vonnahme et al., 2006, Redmer et al., 2001, Campbell et al., 2004) and, therefore, was the fixer used in this project.

Sheep ovaries were washed in PBS pH 7.4 (BDH) solution at room temperature, fixed in 40% v/v Neutral Buffered Formalin (National Diagnostics) solution for 24 hours and then transferred and preserved in 70% v/v ethanol (BDH) until paraffin wax embedding.

2.6.1.2 Tissue processing and embedding

The preparation of tissues for histology requires infiltration and embedding of the specimens in a firm matrix, like paraffin, for microtomy. As paraffin is immiscible with aqueous solutions, fixed tissue samples must be dehydrated prior to paraffin embedding (Wishe et al., 1980).

Ovaries were individually placed in small labelled embedding cassettes (Taab Laboratories) and placed in an automatic tissue processor (Shandon Citadel 2000). The samples were submitted to increasing ethanol concentration (1 hour in 70% v/v ethanol, 1 hour in 90% v/v ethanol, 1 hour and 30 min in the first two washes of 100% ethanol and 1 hour and 45 minutes for the last two 100% ethanol washes) and then placed in CNP30 (Taab Laboratories) for 3 hours, twice. CNP30 is an organic solvent made from a blend of 1,1,1-trichloroethane with perchloroethylene and some additives (Lake, 1980). Paraffin solvents are organic compounds miscible with paraffin at 56 ^{o}C as well as with dehydrating agents at room temperature (Wishe et al., 1980). Samples were then embedded in Paraffin Wax (Paraplast®, Leica, Taab Laboratories) at 60-65 ^{o}C for 4 hours and 30 minutes. This final step impregnates the tissue with molten wax for the final stage, embedding, when tissues are placed in paraffin wax blocks ready to be cut.

Ovaries were placed in stainless steel cassette moulds and covered with molten wax. Whole ovaries were placed with the longest side facing the bottom of the block. Ovaries containing corpora lutea were placed with CL facing down to facilitate cutting. Blocks were allowed to cool before being removed from the mould and then stored at room temperature before sections were cut.

2.6.2 Slide preparation

Prior to sectioning, wax blocks were melted into wooden blocks for support with the tissue surface facing upwards (cutting surface). Six micrometer sections were made using a steel blade microtome (HM 360 Microtome, Microm) and then placed in a water bath at 45 ^{o}C to prevent wrinkling. Sections were placed on glass slides (SuperFrost®Plus, VWR International). These particular slides were used due

to their ability to adhere the sections even under hot conditions for long incubation periods as those found in the *in situ* hybridization protocol. Slides were dried using a hot plate (temperature just below wax melting point) and air dried overnight.

Ovaries from pre-ovulatory animals were serially sectioned whilst ovaries containing CLs only had sections made from the corpora lutea. Two sections were put on each slide and every 25th slide (25, 50, 75, 100, 125th and so on) of serially sectioned ovaries was stained with Hematoxylin-Eosin (HE) to be used for mapping and follicle identification. Ovaries from pre-ovulatory animals (Groups 1 to 4) yielded roughly 300 slides each, resulting in a total of 6,000 serially sectioned slides. Approximately 30 slides were made from each CL containing ovary. These slides were used for both ICH and ISH assays.

2.6.3 Ovary mapping

2.6.3.1 Hematoxylin-Eosin Staining

Slides were placed in CNP30 (Taab Laboratories) for at least 30 minutes for dewaxing and rehydrated through a series of ethanol solutions (100%, 90%, 70% and 50% v/v), 2 minutes in each. Slides were quickly washed in distilled water for 1 minute and placed in Mayer's Haematoxylin (Haemalum-Mayer –Taab Laboratories) for 15 minutes. Slides were then placed under gently running tap water for 5 minutes or until sections appeared blue. Slides were then placed in 1% w/v Eosin (Taab Laboratories) for 3 minutes and washed gently in tap water for 10 seconds following dehydration through graded ethanol solutions (50% and 70% v/v for 1 minute, 90% v/v and 100% for 2 minutes) and placed in CNP30 for at least 5 minutes. Slides were cleaned and mounted using DePex Mounting Medium (BDH) and covered with a glass coverslip (VWR International). The result was a section which had nuclei stained blue with a cytoplasm of various shades of pink. This type of staining allowed easy visualization of follicles and CLs (Figure 9).

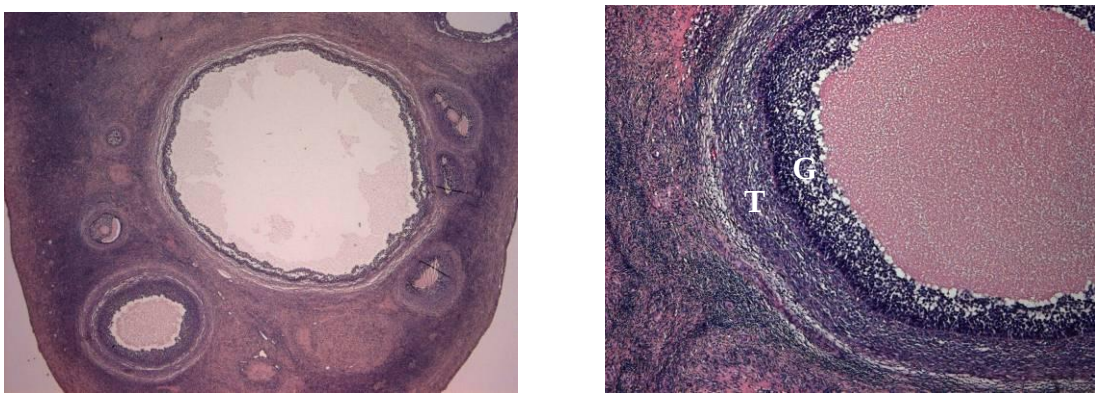


Figure 9. Paraffin-embedded ovarian section from seasonally anoestrous ewes treated with pulsatile GnRH, stained with Hematoxylin-Eosin for mapping. Ovary displaying different size follicles (left) and the different follicular layers (right): granulosa (G) and theca (T). Magnification of 50X and 200X, respectively. Bright field microscopy.

2.6.3.2 Ovary Mapping

Ovary mapping comprised of identifying and measuring follicles bigger than 2 mm in diameter in every 25th slide that had been stained with HE. Slide analysis was done using a bright field microscope with a measuring eyepiece. A picture of the section was drawn and the follicles identified with numbers (Figure 10).

As sheep follicles become gonadotrophin dependent when they reach 2mm in diameter (Driancourt, 2001) follicles smaller than 2mm were not selected for this project. Based on our own observations, the largest follicles detected in animals from Groups 1 to 4 did not exceed 6-7 mm in diameter. As the difference between the dominant follicle and the largest subordinate follicle in sheep is 2-3 mm (Driancourt, 2001), the largest subordinate follicles that could be observed in the experimental animals would have approximately 3-4 mm. Therefore, follicles bigger than 2 mm in diameter were selected for *in situ* hybridization and immunohistochemistry analysis and then further classified into small (2-2.5 mm in diameter) and large (≥2.5 mm in diameter) for statistical analysis.

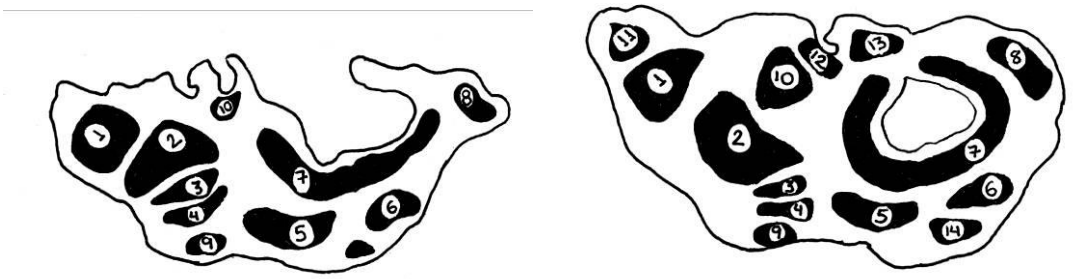


Figure 10. Ovary mapping illustrating follicle distribution on the 50th (left) and 75th (right) Hematoxylin-Eosin stained slide from a progesterone primed seasonally anoestrous ewe treated with pulsatile GnRH slaughtered prior to the LH surge.

2.6.4 Immunohistochemistry assay

Slides of interest were dewaxed in CNP30 (Taab Laboratories) for at least 30 minutes, rehydrated through graded ethanol solutions (100%, 70% and 50% v/v) for 2 minutes each and then washed in deionised water for a further 2 minutes.

Slides were then incubated in 3% v/v Hydrogen Peroxide (Sigma-Aldrich) in Methanol (BDH) for 15 minutes to ensure that any endogenous peroxidase activity was blocked. Peroxidase activity is a common property of many proteins, including haemoglobin, myoglobin and catalases (Boenisch et al., 2001). If endogenous peroxidase activity is not suppressed, peroxidase substrates used at the end of the immunohistochemistry procedure to develop colour may interact with tissue proteins and result in a false positive reaction. Therefore, incubation of slides in Hydrogen peroxide/Methanol solution is done to minimize non-specific results, also known as background staining. This step would not be necessary if Alkaline Phosphatase substrates were used to develop colour at the end of the immunohistochemistry procedure.

Slides were washed twice for 5 minutes in 1x PBS pH 7.4 (BDH) prior to a 10 minute incubation in boiling hot 0.01M Sodium Citrate (Sigma-Aldrich), adjusted to pH 6.0. This step, also known as antigen or epitome retrieval, is done in order to improve immunoreactivity of formalin-fixed tissue antigens. Each antigen may contain from one to many epitopes and each of these may be composed of five or more amino acids. Although some epitopes are formalin-resistant and remain unchanged, some others may undergo substantial changes. These may include loss of

structure and binding capability or cross-linking with unrelated proteins thus "masking" the antigen and making it unavailable for binding (Boenisch et al., 2001). This heat-induced antigen retrieval step allows more epitomes of the protein of interest to be available for binding with the primary antibody to be used.

Slides were dried and sections circled using a water proof pen (ImmEDGE® pen, Vector Laboratories) to avoid spillage of solution.

Sections were incubated in 300 µl of Blocking Solution containing 1% v/v Normal Goat Serum (Vector Laboratories), 20% v/v Avidin D (Vector Laboratories) in PBS pH 7.4 (BDH) for 30 minutes in an humidified chamber. The addition of normal serum will lead to hydrophobic binding between serum proteins and tissue proteins. Due to the similarity of serum proteins and IgG they compete for the same hydrophobic binding sites in the tissue. Therefore when the biotinilated IgG (2^{nd} antibody) is added to the sections there will be less tissue proteins for it to non-specifically bind to. The secondary antibody should bind mostly to the primary antibody thus reducing background staining. Normal serum should be different from the species in which the primary antibody is raised in to further avoid non-specific binding of the secondary antibody,

Slides were drained and probed with 200 µl/section of primary antibody in 20% v/v Biotin (Vector Laboratories) in PBS pH 7.4 (BDH). Antibodies for the different proteins analysed were used at different concentrations and are further specified in Chapters 3 and 4.

Biotin, a vitamin (B7) and coenzyme, can be found distributed in a variety of tissues where it is bound to other proteins. Biotin once in contact with Avidin forms a strong and stable compound. When Avidin is added to the blocking solution it binds to the Biotin found in the tissue. Biotin can only bind to one molecule of Avidin, however, Avidin has 4 binding sites to Biotin (Boenisch et al., 2001). Therefore, incubation with Biotin after incubation with Avidin is essential for the blocking process to be complete. This will reduce any non-specific binding of the Avidin-Biotinilated enzyme complexes (ABC) that are used to enhance the immunohistological response. The use of Avidin/Biotin blocking is essential when ABC detection methods are being used.

For VEGFR-2 assays, a slightly different approach was used for the blocking and primary antibody probing, but which led to similar results. For this specific protein slides were blocked for 30 min in 1% v/v Normal Goat Serum (Vector Laboratories) in PBS, drained, had a drop of Avidin D (Vector Laboratories) added to each section and incubated for 15 minutes, then drained and a drop of Biotin (Vector Laboratories) added to each section and incubated for a further 15 minutes. Slides were then drained and probed with primary antibody diluted in PBS alone.

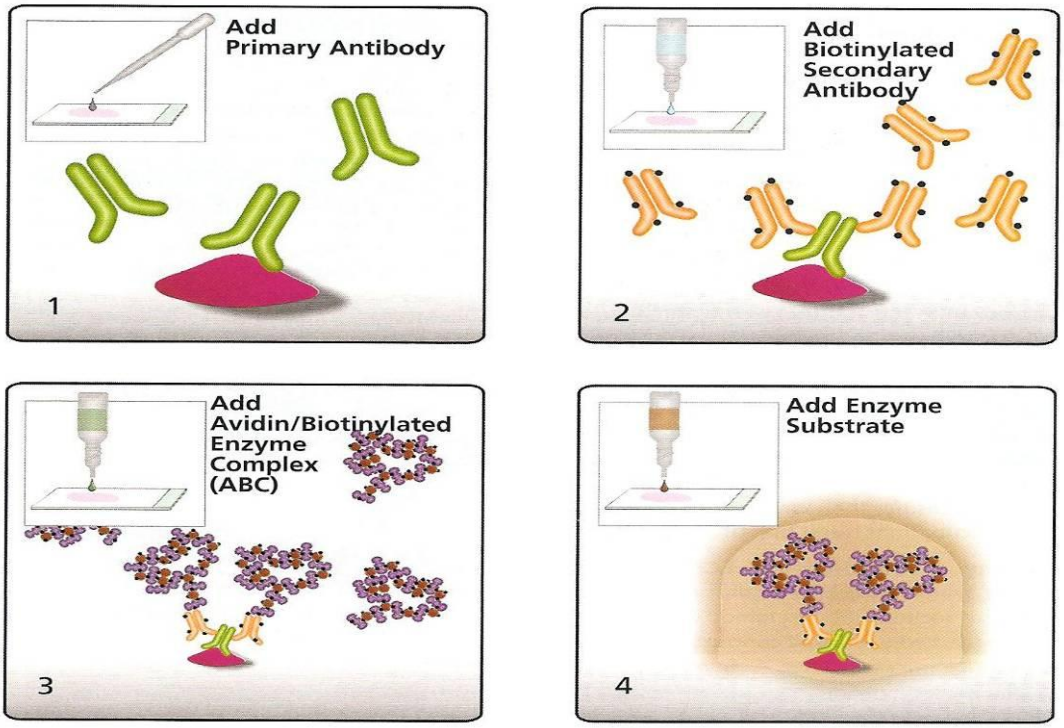
For every 3 positive sections 1 control/negative section was used. The control section was a similar tissue section to the positive ones and was treated in the same way except that instead of primary antibody, Normal Rabbit IgG (Santa Cruz Biotechnologies) was used in the same concentration and diluted in a similar 1% v/v Normal Goat Serum (Vector Laboratories) in PBS solution with biotin (or without in the case of VEGFR-2 assays). Control sections were incubated for as long as the positive ones. Normal IgG used in control slides should be from the same species in which the primary antibody is raised in.

Once the incubation time was over, slides were washed thrice in PBS (BDH) for 5 minutes. This washing step should have been able to remove all excess and unbound primary antibody reducing the chances of a false positive reaction. The Rabbit IgG should not have been able to bind to the tissue as there was no specific antigen or binding site for it and it should have been washed away once the incubation time was over. All sections were then incubated with 200 µl of biotinylated anti-rabbit IgG secondary antibody (ABC Kit, Vector Laboratories) diluted 1:25 in 2% v/v Normal Goat Serum (Vector Laboratories) in PBS for 30 minutes.

The method used in this protocol is the two-step indirect staining method in which an unconjugated primary antibody first binds to the protein of interest in the tissue and a labelled secondary antibody directed to the primary antibody is applied followed by the substrate solution. This procedure is more sensitive than direct methods as several secondary antibodies bind to a number of epitopes of the same primary antibody amplifying the response. The biotynilated secondary antibody used at this step is part of the ABC (avidin-biotin complex) procedure to amplify the positive signal.

Slides were washed twice in PBS (BDH) for 5 minutes to remove excess and unbound secondary antibody. Sections were incubated in 200 µl ABC solution [2% v/v solution A (Avidin DH), 2% v/v solution B (Biotinilated peroxidise enzymes) (ABC Kit, Vector) in PBS] for 30 minutes at room temperature. ABC solution was left to stand at room temperature for 30 minutes prior to being added to the slides. During the 30 minutes pre-incubation period Avidin DH (solution A) binds to the biotynilated peroxidase (solution B) forming a three-dimensional complex. As previously mentioned, Avidin has a strong affinity for Biotin but whilst one molecule of biotin can only bind to one molecule of Avidin, one molecule of Avidin can bind to as many as four biotin molecules. However, due to the molecular orientation of the binding sites in the Avidin molecule, fewer than four molecules of biotin will actually bind, leaving at least one binding site free (Boenisch et al., 2001, Vector Laboratories catalogue 2007-2008).

Once this pre-incubated solution is added to the slides the avidin-biotinylated enzyme complexes bind to the biotinylated secondary antibody found in the tissue through the binding site that was left free in the three dimensional complex. Therefore, to every protein of interest several primary antibodies can be found attached. To every primary antibody several secondary antibodies can be found attached and to every secondary antibody several avidin-biotinylated enzyme complexes can be found attached. This should guarantee a good amplification of the signal obtained in the assay as illustrated in Figure 11.



Vector Laboratories Catalogue 2007-2008

Figure 11. Simplified diagram illustrating the indirect detection method followed by ABC amplification signal used in immunohistochemistry assays.

Slides were washed twice in PBS (BDH) to remove any excess of ABC solution. ImmPACT® DAB (Vector Laboratories) was used as substrate to develop colour. DAB is a diaminobenzidine-based peroxidase substrate which reacts with the enzymes from the ABC solution producing a brown colour in positive slides (Figure 12). Sections were left in contact with DAB for different lengths of time, according to optimized time determined for each protein as detailed in the relevant chapters. Excess DAB was removed by briefly washing slides in deionised water and counterstaining was done by immersing slides in Hematoxylin (Vector Laboratories) for 1 minute. Slides were washed under running tap water for 5 minutes.

After dehydration though a series of graded ethanol solutions (50%, 70%, 100%) slides were placed in CNP30 for at least 5 minutes and mounted with Vectamount Permanent Mounting Medium (Vector Laboratories) using a glass cover slip (VWR International). Slides were air dried overnight and kept away from direct light until analysed by Expression Index scoring using bright field microscopy.

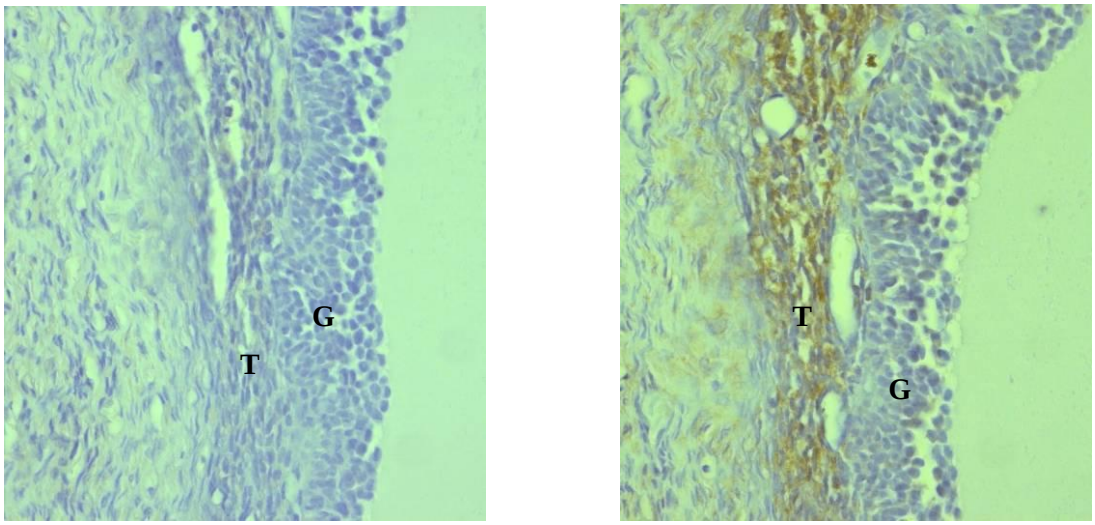


Figure 12. VEGF Immunohistochemistry negative (left) and positive (right) slide following colour development using DAB (brown) and Hematoxilin counterstaining. The granulosa (G) and theca (T) follicular layers are indicated. Bright field microscopy at 400X magnification.

2.7 *In situ* hybridization

In situ hybridization is a technique that allows specific nucleic acid sequences to be detected in morphologically preserved tissue sections. As the name suggests, the detection is done by hybridization of probe and nucleic acid sequence of interest in the exact place that it is found in the cell. The technique provides microscopic topological information and semi-quantitative comparison of DNA and/or mRNA activity (Eisel et al., 2002).

The technique was developed in 1969 by Pardue and Gall and (independently) by John et al. in the same year. At the beginning, radioisotope labels and autoradiography were used to detect hybridized sequences. Nowadays, however, non-radioactive methods of ISH are most commonly employed in association with colorimetric analysis. There are two types of non-radioactive ISH methods: direct and indirect. In the direct method a reporter molecule is bound to the probe thus allowing visualization of probe-target immediately after hybridization. The main advantage of the direct method is the shorter time taken to perform the assay. However, the disadvantages of loss of reporter-probe bond due to harsh hybridization

and washing conditions and the interference of reporter molecule in the hybridization process far outweigh the benefits. Indirect methods require a reporter molecule to be chemically or enzymatically introduced into the probe so that a specific antibody to the reporter molecule can be used. One of the most used systems is the Digoxigenin (DIG) System (Eisel et al., 2002) (see section 2.7.1.11)

The significant advantage of ISH when compared to methods like Southern and Northen Blotting is that it permits the localization of the hybridization material within a cell or tissue. The major disadvantage of ISH is that maintaining the morphology and structures of the cell potentially introduces inhibitory elements. To overcome such conditions pre-hybridization treatment of tissues are essential (Baumgart et al., 2001)

2.7.1 Riboprobe synthesis

RNA probe sysnthesis involves various steps which follow on from one another. These steps include: (1) RNA extraction, (2) Complimentary DNA (cDNA) synthesis, (3) Polymerase Chain Reaction (PCR), (4) Gel extraction, (5) Ligation, (6) Transformation, (7) Bacterial selection, (8) Bacterial growth, (9) DNA extraction, (10) Restriction Digest and Linearization and (11) Probe synthesis and label.

Not all steps were performed in this project as bacteria containing the plasmid of interest were supplied. Thus only steps 2.7.1.8 to 2.7.1.11 were performed for the work in this project. Steps 2.7.1.1 to 2.7.1.7 were performed in our laboratory by Dr Waliul Chowdhury, and will not be discussed in detail in this thesis.

2.7.1.1 RNA extraction

In order to make riboprobes that will detect ovine mRNA, sheep tissues such as liver, ovary and muscle had their RNA extracted. The extraction process basically consists of homogenizing the tissue and RNA isolation using phenol ethanol and a number of centrifugation steps. The total RNA extracted is composed of messenger RNA (mRNA), Ribosomal RNA (rRNA) and Transporter RNA (tRNA).

2.7.1.2 Complimentary DNA synthesis

A DNA sequence is synthesised based on the extracted mRNA. Incubation of RNA, nucleotide bases (A, T, G and C) and M-MLV enzyme at 37 °C for 1 hour results in the production of a strand of DNA complimentary to the RNA available.

2.7.1.3 Polymerase Chain Reaction

Messenger RNA sequence of the protein/factor of interest is identified and PCR primers are designed so that only specific cDNA is amplified. This process guarantees that the cDNA from the mRNA of interest is present in a much greater number than any other cDNA.

2.7.1.4 Gel extraction

PCR samples are run on agarose gel and, based on the number of base pairs of the cDNA of interest, the band of the product is identified on the gel and extracted using a series of buffers and centrifugation.

2.7.1.5 Ligation

This process consists of inserting the extracted cDNA into a linear vector. When the cDNA is added the DNA binds to both ends of the vector forming a circular complex called a plasmid. Plasmids are then ready to be inserted into bacteria and replicated.

2.7.1.6 Transformation

Competent *Escherichia coli* are used in this step. These bacteria have already perforated membranes enabling substances, such as the plasmid, to pass into the cell. PGEM-T Easy Vector (Promega) containing the DNA sequence of interest was inserted in the bacteria used in this project.

2.7.1.7 Bacterial selection or blue and white screening

The bacterial cells are spread on agar plates containing a selective broth. Following overnight incubation both blue and white bacterial colonies can be identified. Blue colonies are those that contain the vector but not the DNA of interest. The white colonies, on the other hand, contain the plasmid. These white colonies are picked from a plate, grown in broth and kept at -80 °C for further use.

2.7.1.8 Bacterial growth

Approximately 10 ml of autoclaved 25% w/v LB Broth (Merk) in water was pipetted into a 50 ml sterile falcon tube and left to reach room temperature for approximately 30 minutes. One tube was used as a control and two other tubes to grow the bacteria of interest. To each tube (except control) 50 µl of the bacterial culture containing the plasmid of interest was added and all tubes were incubated in a shaker at 37 °C for 6 hours.

After this first incubation period, the control and bacteria cultures were added to a sterile glass conical flask containing 90 ml of LB broth. By this stage it was possible to observe if there was bacterial growth in the falcon tubes by the opaque aspect of the broth. The flasks were covered with aluminium foil and incubated in a shaker, at 37 °C, overnight.

If by the next day the control flask had signs of contamination the cultures would be disposed of and procedure repeated. However, if the control flask was clear, cultures would be used for cDNA extraction. In order to have a stock of these bacteria containing the plasmid of interest, 500 µl of bacteria culture in broth was added to 500 µl of glycerol (BDH) and stored in 2 ml microtubes at -20 °C.

2.7.1.9 DNA extraction

Complimentary plasmid DNA extraction was performed using Qiagen® Plasmid Kit (Qiagen). The protocol and reagents used were those provided with the product by the manufacturer.

Bacterial culture was put into 50 ml falcon tubes and the bacteria cells harvested by centrifugation at 6,000 x g for 15 minutes at 4 °C. All traces of supernatant were carefully removed by inverting the tube. Bacterial pellets were re-suspended in 4 ml of Buffer P1 with RNAse A by vortexing until a homogeneous solution was obtained. To each tube 4 ml of Buffer P2 was added and mixed gently by inverting the tubes 4-6 times. Lysis reaction was performed for 5 minutes at room temperature. The precipitation of genomic DNA was achieved by adding 4 ml of chilled Buffer P3 and incubating in ice for 15 minutes, after gently mixing. A fluffy white material formed and the lysate became viscous. The precipitated material contained genomic DNA, proteins, cell debris and SDS.

Samples were centrifuged at 20,000 x g for 30 minutes at 4 °C. Supernatant containing plasmid DNA was removed and transferred to a clean falcon tube. Samples were centrifuged at 20,000 x g for a further 15 minutes. Supernatant was removed and kept for filtration.

Qiagen Tips were equilibrated by applying 4 ml of buffer QBT and column was allowed to empty by gravity flow. Supernatant containing plasmid DNA was applied to the Qiagen Tip and allowed to enter the resin by gravity flow. If the process is successful cDNA should be completely bound to Qiagen resin in tip. Tips were washed twice with 10 ml of Buffer QC to remove the majority of contaminants in the plasmid DNA preparation. Using new tubes, DNA was eluted by adding 5 ml of Buffer QF to the Qiagen Tip. DNA was precipitated by adding 10.5 ml of room temperature isopropanol to the eluted DNA. Samples were mixed and centrifuged at 20,000 x g for 30 minutes at 4 °C. The supernatant was carefully removed and the DNA pellet was further washed with 5 ml of 70% v/v ethanol and centrifuged at 20,000 x g for 10 minutes. The supernatant was removed and the pellet air-dried for 5-10 minutes prior to dilution in 100 µl of Nuclease Free Water (NFW, Ambion). This solution will be referred as Midi-prep solution from now on. Tubes were labelled and kept at -20 °C until needed for linearization.

2.7.1.10 Restriction digest and linearization

Prior to sample preparation, the work bench was disinfected with 70% v/v ethanol (BDH) and RNAse free cleaning wipes (RNAseZap®, Ambion). The reagents used in this protocol were thawed in ice prior to being used.

Two 200 µl RNA free microtubes were used for cDNA linearization. To each tube 59 µl of NFW (Ambion), 1 µl of BSA (10 µl/ml – Promega) and 10 µl of Midi-prep solution were added. One of the tubes was used as a precursor for producing anti-sense (AS, positive) probes whilst the other as precursor for sense (S, negative) probes.

AS probes are made from a complimentary sequence to the mRNA of interest found in the tissue. Therefore, under hybridization conditions it binds to the mRNA and this results in a positive response. The Sense probe, on the other hand, has exactly the same sequence as the mRNA found on the tissue and does not bind to the sequence of interest, thus working as a negative control to monitor hybridization procedure.

To AS microtubes 10 µl of Buffer A and 10 µl of restriction enzyme Apa I (Promega) were added. The solution was mixed thoroughly by pippetting up and down several times, and the microtubes placed in RNAse-free water bath at 37 °C overnight. To S tubes 10 µl of Buffer D and 10 µl of restriction enzyme Sal I (Promega) were added and the solution incubated as stated above.

Restriction enzymes recognize a short, specific DNA sequence. They cleave double stranded DNA at specific sites (restriction sites). Each enzyme has a specific requirement for optimal activity, thus the use of different reaction buffers for each enzyme (Product Information sheet, Promega). Apa I enzymes cleave the DNA of interest on one side whilst Sal I cleaves it at the opposite side.

After 16 hours incubation the cDNA concentration in the tubes was determined by 1% w/v agarose gel/ethidium bromide electrophoresis. For each gel, 1.5 g agarose (BDH) was added to 150 ml TAE (Promega). The mixture was microwaved for 2-3 minutes until the agarose dissolved. The flask was left to cool down at room temperature for approximately 20 minutes when 10 µl of ethidium bromide (Sigma-Aldrich) was added. Ethidium bromide is a nucleic acid intercalating agent

commonly used as DNA/RNA stain for agarose gel electrophoresis (Sigma product information sheet). The solution was mixed and left to cool down at room temperature for a further 10 minutes.

Agarose mixture was poured into an RNAse-free horizontal gel cassette and left to polymerize for 30 minutes. Once set, the gel was placed in chamber and covered with 1x TAE buffer prior to sample loading.

Apa and Sal testing samples were prepared by adding into 200 µl RNAse-free microtubes 2 µl of Apa or Sal linearized solution, 1.5 µl Blue/Orange 6x loading dye (Promega) and 6.5 µl NFW. These test tubes were kept on ice until loading, and the original Apa and Sal solutions were stored at -20 °C until needed.

To the first well of the agarose gel, 5 µl of 100bp DNA ladder (Promega) was added and 10 µl of test samples were loaded into the remaining wells. Electrophoresis was performed at 125v, 400mA for 50 minutes. DNA concentrations were roughly estimated by placing the agarose gel under an Ultra-violet (UV) box and comparing intensity of the bands found in the samples with those of the 500bp band in the ladder (5 µl ladder contains 150 ng of 500pb band), as illustrated in Figure 13.

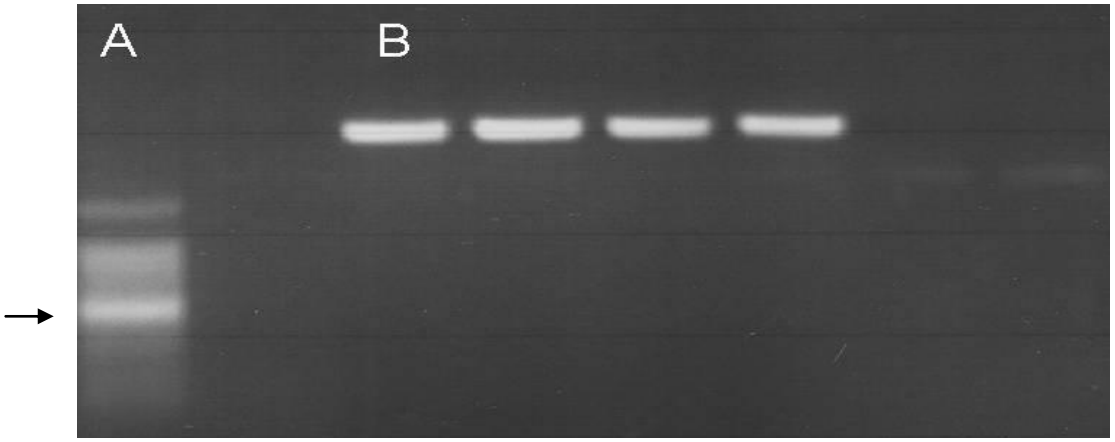


Figure 13. Agarose/ethidium bromide gel image used to estimate Angiopoietin-2 cDNA concentration in four Apa samples (B) based on the 500bp band (arrow) from the DNA ladder (A).

The Apa bands illustrated in the figure above showed fluorescence intensity over twice that of the 500pb band. As the intensity of the 500bp band corresponds to 150 ng, the total cDNA concentration of the Angiopoietin-2 Apa bands was estimated at approximately 400 ng. Considering 2 µl of solution was used to estimate cDNA concentration, the final concentration of these Ang-2 Apa solutions was 200 ng/µl.

From this point onwards the linearized cDNA solutions will be reffered to as either Sal (for S probes) or Apa (for AS probes).

2.7.1.11 Probe synthesis and label

This final step in probe making was done using Megascript SP6 polymerase kits (Ambion) for AS probes and Megascript T7 polymerase kits (Ambion) for S probes. Each kit contained ATP, CTP and GTP nucleotides, unlabelled UTP, 10x Reaction Buffer, Nuclease Free Water, Polymerase Enzyme mix, DNAse and Lithium Chloride (LiCl). The DIG-11-UTP label used was supplied by Roche (Roche).

During this step the DNA of interest is amplified time and time again. However, rather than synthesising DNA the nucleotides used result in RNA being synthesised instead. RNA probes are generated by *in vitro* transcription from a linearized template (linearized cDNA). A promoter for RNA polymerases must be available on the vector DNA containing the template of interest. The synthesised transcripts are an exact copy of the sequence from the promoter site to the restriction site used for linearization. Riboprobes are inherently single-stranded (Eisel et al., 2002).

The DIG labelling method is based on a steroid isolated from *Digitalis purpurea* and *D. lanata*. As these plants are the only natural source of digoxigenin, the antibody used in the indirect method does not bind to other biological materials. The DIG-labelled UTP nucleotides can be incorporated randomly by DNA or RNA polymerase, however, for most reactions DIG labelled nucleotide is incorporated every 20 to 25th nucleotide (Eisel et al., 2002).

All reagents were removed from the -20 °C freezer and thawed in ice before being used. For every 20 µl of probe labelling solution, 1-3 µg of linearized cDNA was used. The volume of Sal or Apa solution to be added was calculated based on the

cDNA concentration determined in the previous step. The concentrations of the reagents used to prepare 20 µl of AS and S probe solutions are detailed in Table 3.

Table 3. Reagents concentration for making a 20 µl solution of sense or anti-sense probe for *in situ* hybridization assays.

Reagents (µl)	Sense	Anti-sense
ATP	2	2
CTP	2	2
GTP	2	2
unlabelled UTP	1.3	1.3
DIG-11-UTP	5.2	3.5
10x Reaction Buffer	2	2
Enzyme mix	2	2
cDNA	1-3 µg	
NFW	make up to 20 µl	

Reagents were mixed thoroughly and placed in a water bath at 37 °C for 6 hours or overnight. Tubes were then removed from the water bath and placed on ice for 5-10 minutes. To each 20 µl solution 1 µl of DNAse was added and tubes placed in water bath, at 37 °C, for 30 minutes. This step should ensure that the template DNA used to make the riboprobe is digested leaving only RNA probes in the solution.

To every tube 30 µl of NFW, 25 µl of LiCl and 150 µl of ice cold 100% ethanol (BDH) was added. Microtubes were placed in a -20 °C freezer overnight for precipitation of riboprobes into a white pellet. The next day, samples were removed from the freezer and immediately centrifuged at 13,000 rpm for 30 minutes at 4 °C. The supernatant was discarded and the pellet washed with 150 µl of ice cold 70% v/v ethanol (BDH) followed by 10 minutes centrifugation at 13,000 rpm at 4 °C. The supernatant was discarded and the pellet allowed to air dry in ice for a further 5-10 minutes. The pellet was re-suspended in 20 µl of NFW and 1 µl of the solution was taken to test the probe concentration. Probes were kept at -20 °C until needed for ISH assays.

To the 1 µl of probe solution 7 µl of NFW and 2 µl of Blue/Orange dye (Promega) were added. Concentration was estimated by agarose gel/ethidium bromide electrophoresis followed by UV fluorescence of gel (Figure 14), as described previously.

All probes were tested in an ISH assay prior to being used on experimental slide assays.

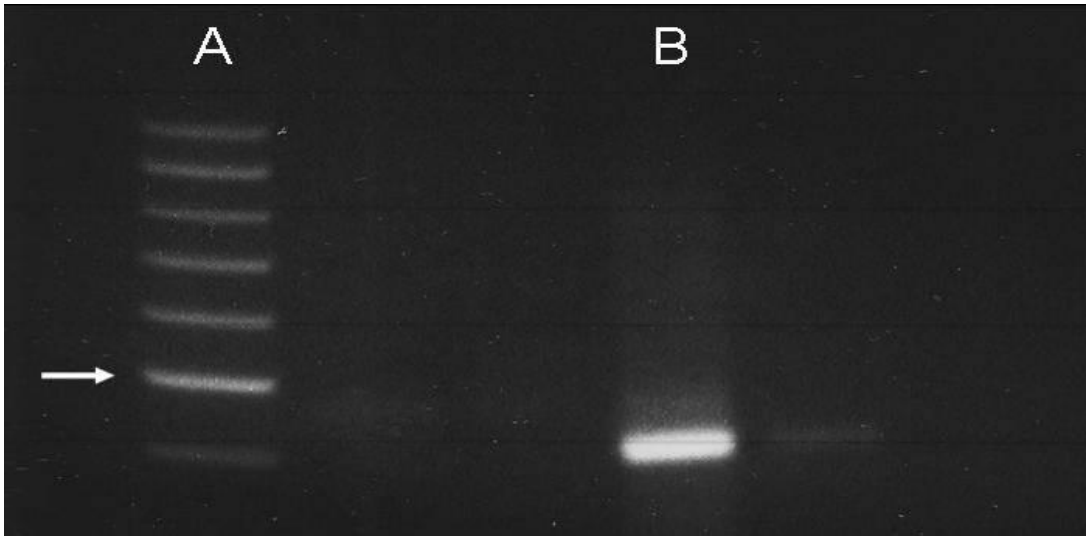


Figure 14. Agarose/ethidium bromide gel image used to estimate Angiopoietin-2 anti-sense probe concentration (B) based on the 500pb band (arrow) from the DNA ladder (A).

2.7.2 *In situ* hybridization assay

Ovarian slides were placed in racks and dewaxed in CNP30 (Taab Laboratories) or Histosol (National Diagnostics) for approximately 30 minutes followed by rehydration through a series of ethanol solutions (100%, 70%, 50% and 25% v/v) for 2 minutes. Slides were placed for 5 minutes in autoclaved deionised water prior to 5 minutes incubation in Neutral Buffered Formalin (National Diagnostics). This step should help prevent sections becoming loose from slides further along the assay.

Slides were washed twice in 1x PBS pH 7.0 (BDH) for 5 minutes and then placed in boiling hot 0.01 M Sodium Citrate (Sigma-Aldrich) for 35 minutes. Slides were then washed for 2 minutes in PBS and placed in 1mM EDTA (Sigma-Aldrich) in PBS for 5 minutes. EDTA inactivates metal dependent enzymes which could damage DNA/RNA (Eisel et al., 2002). Slides were transferred to a solution comprising 1mM EDTA, 0.1% w/v Proteinase K (Sigma-Aldrich) in PBS and were kept at 37 °C, using a water bath, for 10 minutes. This protease treatment serves to increase target

accessibility by digesting the protein surrounding the nucleic acid, thus allowing the designed probe to reach the mRNA of interest more easily (Eisel et al., 2002).

Slides were incubated in 0.2% w/v Glycine (Sigma-Aldrich) in PBS for 5 minutes at room temperature to stop the Proteinase K reaction. Incubation in 20% v/v Acetic Acid (BDH) in Methanol (BDH) was performed at room temperature for 40 minutes. The acid treatment leads to protein extraction and partial hydrolysis of the target improving the signal-to-noise ratio (Eisel et al., 2002)

Slides were washed in PBS for 5 minutes, drained and sections individually circled using ImmedgePen® (Vector Laboratories). This was performed to avoid loss of solutions that are placed directly on the sections, such as hybridization buffer, and also avoid AS probes mixing with S probes due to direct contact of solutions from adjacent sections. Hybridization buffer consisting of 10% w/v Dextran Sulphate (Sigma-Aldrich), 50% v/v Deionised Formamide (QBiogene), 8.4% v/v autoclaved deionized water, 25% v/v of 2x SSC solution (17.5% w/v NaCl and 8.82% w/v Sodium Citrate in water, pH adjusted to 7.0), 1% v/v denaturated DNA from herring sperm (Sigma-Aldrich) and 5% of Denhart's solution (Sigma-Aldrich) was heated in a water bath at 55 °C.

Both S and AS probes were diluted in Hybridization Buffer (HB) to a final concentration of 1 ng/µl. Approximately 50 µl of HB was added to each section resulting in 50 ng probe/section. Hybridization was performed in a humidified chamber at 60 °C overnight. Incubation times were optimized for each probe/mRNA of interest and further information can be found in the following chapters. Hybridization depends on the ability of denaturated RNA (in tissue) to reanneal with complimentary strands (AS probes) just below their melting point. Renaturation of DNA/RNA occurs roughly between 16-32 °C (Eisel et al., 2002). Incubation of slides at 60 °C allows target RNA to remain denaturated and allow the probe to bind to it. Also, the use of high temperatures reduces the risk of mismatching of base pairs, thus increasing hybridization rate and stability of hybrids. Monovalent cations (such as sodium ions) interact with nucleic acids causing the electrostatic repulsion between strands to decrease with increasing salt concentration, therefore, the higher the salt concentration the more stable the target-probe hybrid is bound to be (Eisel et

al., 2002). The high levels of sodium found in the HB should contribute towards stabilization of target-probe complex.

On the following day HB was drained and slides washed twice in warm (60 °C) 2x SSC solution. During hybridization, duplexes form not only between perfectly matched sequences but also between imperfectly matched ones. It is important to remove background associated with non-specific hybrids and this can be achieved by washing slides in a diluted solution of salt at a relatively high temperature (Eisel et al., 2002).

Slides were incubated in 0.01% w/v RNAse (ABgene) in 2x SSC at 37 °C for 30-35 minutes. This digests any unbound RNA probe removing high background and non-specific signals. Slides were further washed once at room temperature with 2x SSC for 2 min and twice in warm (60 °C) 2x SSC for 10 minutes followed by 10 minutes in warm (60 °C) 1x SSC.

Slides were washed for 5 minutes in TBS containing 0.8% w/v NaCl (Sigma-Aldrich), 0.02% w/v Potassium Chloride (KCl, BDH), 0.3% w/v tris-base (BDH) in water, pH adjusted to 7.4. Slides were placed for 5 minutes in AP1 solution [0.1M tris (BDH) HCl added to adjust pH to 7.5, 0.1M NaCl (Sigma-Aldrich), 2mM Magnesium Chloride ($MgCl_2$, Sigma-Aldrich) in water]. Slides were drained and placed on tray. Approximately 500 µl of 3% w/v BSA (Sigma-Aldrich) in AP1 was added to each section and allowed to incubate for 30-40 minutes. The BSA binds to the tissue in almost every place except those sites that already contain the hybrid. This step should significantly reduce non-specific binding of antibody.

An Alkaline Phosphatase labelled anti-digoxigenin antibody (Roche) was diluted in 3% w/v BSA (Sigma-Aldrich) in AP1 to a final concentration of 1:500. Slides were drained and 100 µl of antibody solution was added to each section and left to incubate for 2 hours. As the digoxigenin used in labelling the probes can only be found in the *Digitalis spp* plants, the anti-digoxigenin antibody is extremely specific and should not bind to any other biological material.

Slides were drained and washed 3 times in AP1 for 5 minutes followed by a 5 minute wash in AP2 (0.1M tris HCl added to adjust pH to 9.5, 0.1M NaCl and 0.01M $MgCl_2$ in water).

Colour developing solution was prepared using 0.35% v/v NBT (Roche Applied Science), 0.35% v/v BCIP (Roche Applied Science), 1% v/v Levamisole (Vector Laboratories) in AP2. Approximately 500 µl of colour developing solution was added to each section and slides were left to develop colour overnight in the dark. Developing colour times were optimized for each probe/mRNA of interest and further details can be found in the following chapters. The Alkaline Phosphatase from the antibody reacts with the NBT/BCIP to form a stable precipitate. Colorimetric detection methods have the advantage of being easy to locate within the tissue, high sensitivity and good stability of precipitates. Levamisol is added to the solution to inactivate any endogenous alkaline phosphatase activity. This step is performed more out of precaution than necessity as most alkaline phosphatase activity is usually lost during hybridization (Eisel et al., 2002).

On the next day slides were washed for 5 minutes in TBS to remove excess colouring solution. Slides were mounted using warm glycerol gelatin (Sigma-Aldrich) and glass coverslips. Slides were kept protected from direct light to prevent fading of colouring. An illustrative image of an ovarian slide after *in situ* hybridization can be seen in Figure 15.

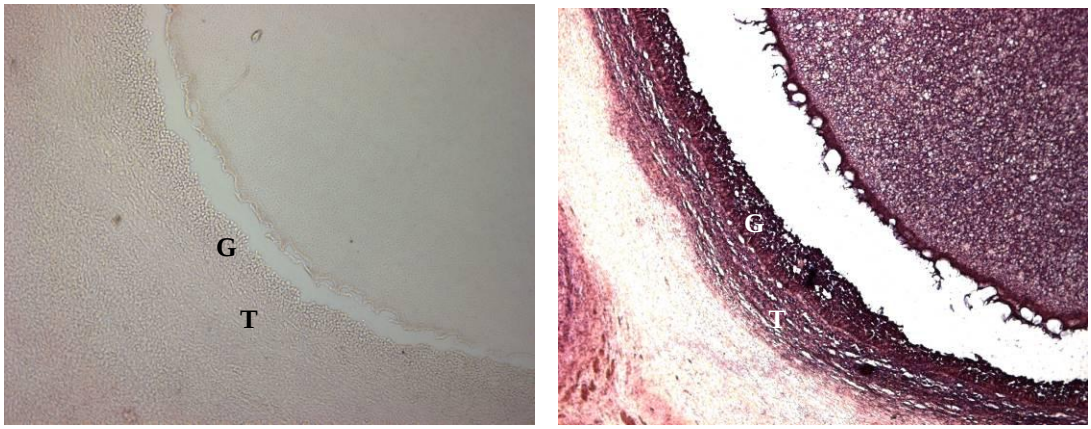


Figure 15. Follicular image from seasonally anoestrous ewe treated with small-dose multiple injections of GnRH with progesterone priming, following *in situ* hybridization with VEGF sense (left) and anti-sense probes (right). The granulosa (G) and theca (T) layers are identified. Bright field microscopy at 200X magnification.

2.7.3 Analysis of slides

Slides from ISH and ICH assays were analysed visually under bright field microscopy at 200 and 400x magnification for better visualization of luteal and follicular cell layers, respectively. The granulosa, theca interna and theca externa cell layers were analysed separately. Each follicle was analysed in 3 different sections, at 3 randomly selected areas in each section, resulting in a total of 9 areas analysed per cell layer/follicle. CL were analysed in 3 different sections at 3 randomly selected areas.

2.7.3.1 Expression Index scoring and Visual Appraisal method

For every cell layer the percentage of positively stained cells and the intensity of the staining were recorded. As intensity of the staining was categorized into weak (1), medium (2) or strong (3), as illustrated in Figure 16, therefore, the proportion (percentage) of the specifically stained cells to which each intensity staining corresponded to, was also recorded.

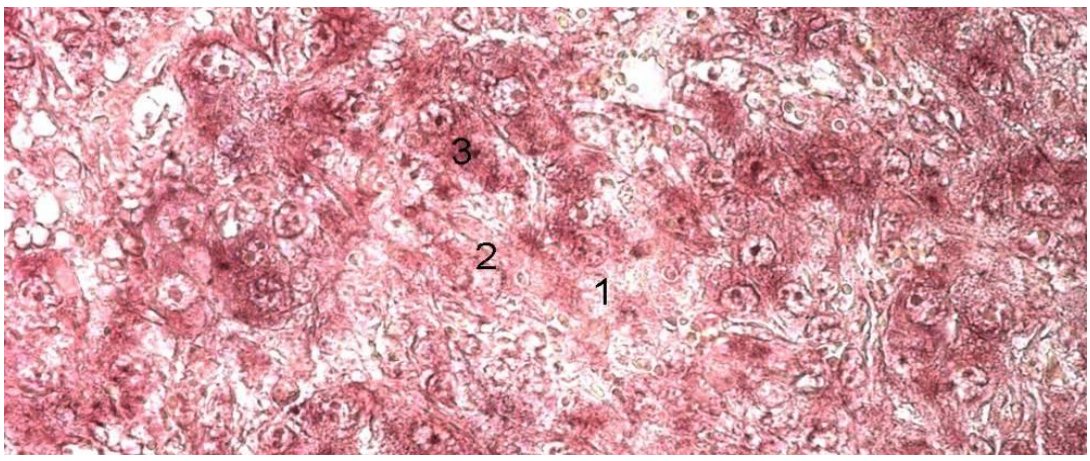


Figure 16. *In situ* hybridization illustrating weak (1), medium (2) and strong (3) intensity staining of mRNA expression for Angiopoietin-2 in the *corpus luteum* of seasonally anoestrous ewes treated with pulsatile GnRH. Bright field microscopy at 400X magnification.

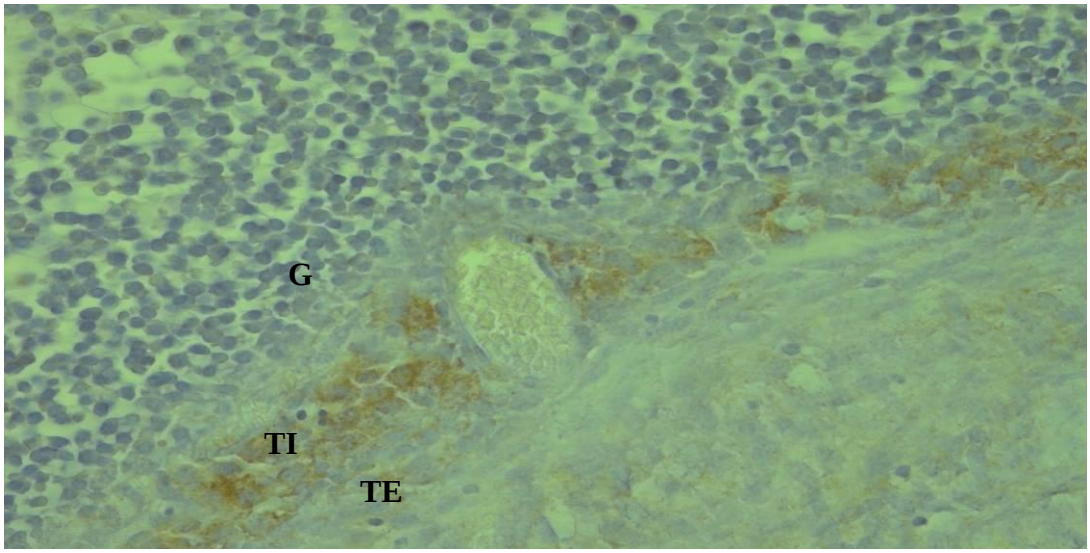
The percentage of a specific staining was multiplied by its value (1, 2 or 3) and an Expression Index for each cell layer was calculated based on the values for each stain intensity (Figure 17). The Expression Index (EI) for each cell layer / section was calculated as follows:

EI = % total stain **x** [(1 **x** % weak) **+** (2 **x** % medium) **+** (3 **x** % strong)]/100

For example, if the total area stained in a section is 80% and from that weak staining corresponds to 60% whilst medium and strong correspond to 20% each then the EI would be:

EI = 80 x [(1x60)+(2x20)+(3x20)] /100 = 80 x (60 + 40 + 60)/100 = 128

The Mean Expression Index of each cell layer was calculated based on the mean values obtained from all areas of all sections (9 values). Both ISH and ICH analysis were done using Expression Index scoring.



Layer	% staining (0-100)	Intensity						Expression Index
		1	1xn	2	2xn	3	3xn	
G	0	-	-	-	-	-	-	0
TI	90	20	20	40	80	40	120	198
TE	30	80	80	20	40	-	-	36

Figure 17. Example of semi-quantitative assessment of VEGF immunostaining in different follicular layers (G = granulosa, TI = theca interna, TE = theca externa) in a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH without progesterone priming . The Expression Index is calculated based on the overall percentage of staining and the different intensities of staining where 1 = weak, 2 = medium and 3 = strong staining. Bright field microscopy at 400X magnification.

2.7.3.2 Visual Appraisal and Image Software Analysis methods

The Visual Appraisal method (Expression Index scoring), although relatively subjective, proved to be a much quicker method of analysis than any done with the aid of a computer software analysis system, facilitating analysis of very large numbers of slides within the current study.

However, the Visual Appraisal method of analysis was compared with results obtained using image analysis software, by correlation and a regression analysis. Pictures were taken from a variety of follicles, and 50 areas of positive staining representing a range of staining intensities were selected for analysis. These areas were initially scored using the Expression Index scoring system and then analysed

using the image analysis software ImageScope version 9.1.19.1571 (Aperio Technologies).

Both Pearson's correlation and regression analysis were performed using SPSS® statistical software programme version 15.0 (SPSS Inc., Chicago, Illinois, USA). There was a significant ($P<0.01$) correlation of 0.915 between the Visual Appraisal and the Image Software Analysis methods and a regression coefficient (R^2) of 0.837 was obtained (Figure 18). These results clearly indicate a very high degree of homology between the subjective Expression Index scores and those obtained from the use of relatively more objective image analysis software, confirming that the more rapid Visual Appraisal method is suitable for quantitative estimation of ICH and ISH results.

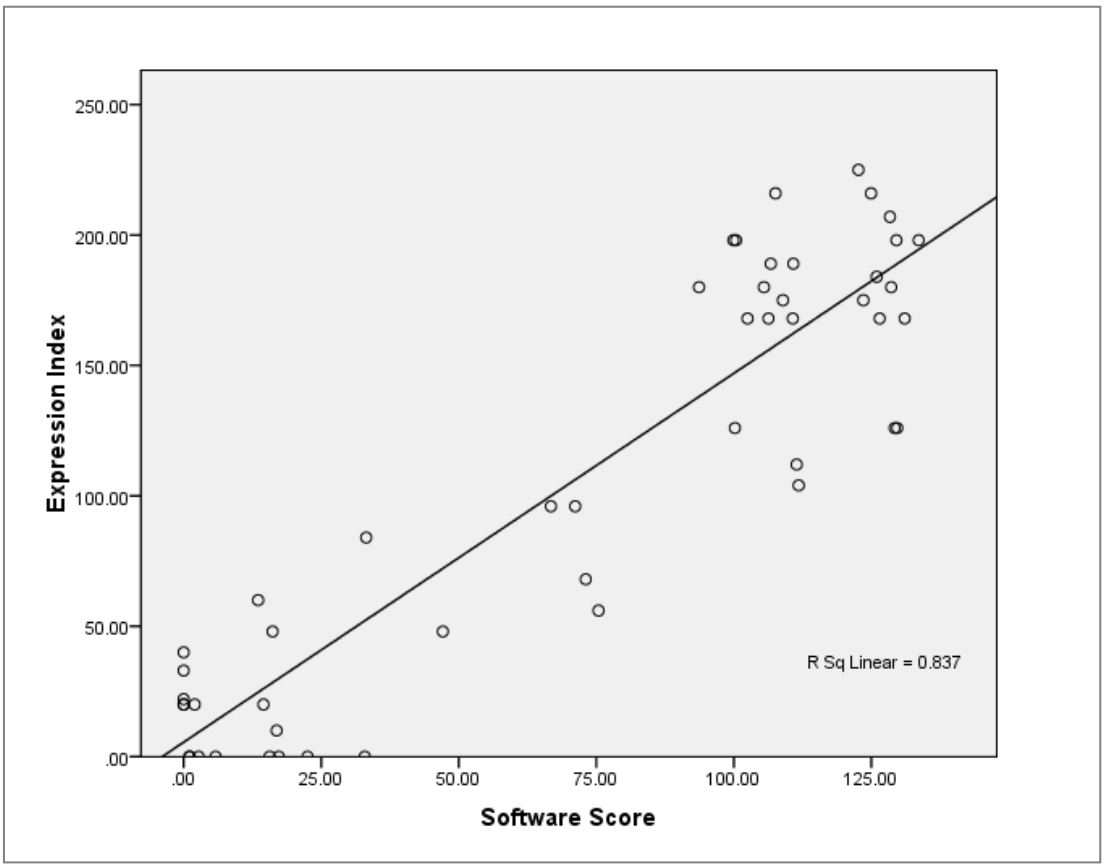


Figure 18. Regression coefficient graph illustrating the relationship between the Visual Appraisal (Expression Index, Y axis) and the Image Software Analysis (Software Score, X axis) methods used to analyse 50 ovarian images following immunohistochemical detection of VEGF using DAB substrate.

CHAPTER 3

Protein and mRNA expression of angiogenic factors in follicles before and after the pre-ovulatory LH surge

3.1 Introduction

Ovine follicular vascular development initiates during the mid to late secondary stage when the stromal cells that surround the follicle develop into the theca interna and theca externa layer (Stevens and Lowe, 1993, Fortune, 2003). Under appropriate stimulation angiogenesis continues throughout follicular growth until ovulation (Kaczmareck et al., 2005).

Ovine follicles become gonadotrophin dependent when they reach approximately 2 mm in diameter (Driancourt, 2001). FSH stimulates initial follicular development, but as the follicle matures there is a switch from FSH to LH dependency (Hunter et al., 2004). FSH induces general follicular development and the expression of LH receptors in the granulosa cells of mature follicles (Stabenfeldt and Edquist, 1996) while LH stimulates dominant follicle growth and indirectly influences oestrogen production (Driancourt, 2001), which in turn stimulates GnRH pulses that induce the pre-ovulatory LH surge. Prior to the LH surge, locally produced inhibitors prevent the granulosa cells from becoming prematurely luteinized. However, after the LH surge the granulosa cells become responsive to LH and begin to secrete progesterone and intrafollicular substances that cause follicular rupture and ovulation (Stabenfeldt and Edquist, 1996).

It is widely accepted that pulsatile GnRH treatment alone is sufficient to cause ovulation in seasonally anoestrous ewes (Stabenfeldt and Edquist, 1996, Southee et al., 1988b, McLeod et al., 1983, McLeod et al., 1982a). However, unless primed with progesterone prior to GnRH-induced ovulation most ewes develop a defective CL (Husein and Kriddli, 2003, Haresign et al., 1996, Southee et al., 1988a).

As vascularity and normal tissue function are closely related (Kaczmareck et al., 2005, Berisha and Schams, 2005, Redmer et al., 2001) it is possible that the lack of progesterone priming may lead to inadequate vascularity of the newly formed CL and consequently result in defective luteal function. Although limited vascularity may not interfere with the capability of the dominant follicle to ovulate it may influence its development and cell layer constitution.

Angiogenesis is essential for tissue growth and development and is a critical component in follicular and luteal function and development (Tamanini and De

Ambrogi, 2004). Antral follicles show two concentric vascular networks, an inner network in the theca interna and an outer network within the theca externa layer. The theca interna vascular network is, however, the main source of nutrients for the follicle (Mattioli et al., 2001).

Follicular angiogenesis is mainly regulated by the VEGF-Angiopoietin system. VEGF is essential for follicular development regulating theca layer vascularity, granulosa cell proliferation, antral formation and steroidogenesis (Hunter et al., 2004, Ferrara et al., 2003a). Both theca and granulosa cells are able of expressing VEGF, however, as the follicle develops the granulosa layer becomes the main source of VEGF (Hunter et al., 2004, Robinson et al., 2007). VEGFR-2, on the other hand, is found mainly in endothelial cells of the theca layer (Wulff et al., 2001a, Hunter et al., 2004).

VEGF production varies according to follicular size. Small follicles have significantly lower levels of VEGF than large ones (Mattioli et al., 2001). VEGFR-2 expression in the theca layer seems to follow a similar pattern to that of VEGF protein (Tamanini and De Ambrogi, 2004). LH has been shown to stimulate VEGF production (Driancourt, 2001) and in the peri-ovulatory period VEGF levels increase markedly in follicular fluid and can be detected in granulosa derived luteinized cells (Ferrara et al., 1992, Tamanini and De Ambrogi, 2004).

Angiopoietins act in association with VEGF to promote blood vessel formation, maturation or regression (Davis et al., 1996). Both theca and granulosa cells are able to express Ang-1 and Ang-2 mRNA while TIE-2 mRNA expression seems to be restricted to the theca cells. Ang-1 expression does not seem to vary between developing and mature follicles. On the other hand, Ang-2 is found to be significantly lower in mature than developing follicles (Hayashi et al., 2003, Shimizu et al., 2007, Tamanini and De Ambrogi, 2004). It has been shown that Angiopoietins are associated with the changes in follicular capillaries at the time of the LH surge. Ang-1 mRNA is highly expressed before the LH surge followed by a significant decrease at the time of the LH surge. A slow and constant rise is, however, observed soon after the LH surge. Although Ang-2 levels are steady throughout most of follicular development, it reaches its lowest level soon after the LH surge. The Ang-2:Ang-1 ratio which is increased at the time of the LH surge gradually declines by

ovulation time (Shimizu et al., 2007). TIE-2 expression remains unchanged throughout follicular development (Hayashi et al., 2003) but reaches its highest just prior to the LH surge (Shimizu et al., 2007).

CL formation consists of changes in the theca and granulosa cells of the pre-ovulatory follicle that occurs after the LH surge (Kaczmareck et al., 2005). Under the influence of LH theca and granulosa cells differentiate into small and large luteal cells, respectively, forming the steroidogenic parenchyma of the newly developed CL (Berisha and Schams, 2005, Schams and Berisha, 2004, Sawyer, 1995). In order to meet the demands of the extensive changes that take place in the follicular cell layer after the LH surge, a well established vascular supply is essential for normal luteal development (Robinson et al., 2007).

Around the time of the LH surge the basal membrane between the theca interna and the granulosa layer dissolves and theca capillaries expand and invade the avascular granulosa to form a dense network (Shimizu et al., 2007). Therefore, adequate development of follicular vascularity may be the key for an adequate luteal blood supply to be established. Furthermore, poor follicular blood supply could affect the initial stages of luteinisation of the theca and granulosa layers that occur soon after the LH surge and throughout early luteal development (Stabenfeldt and Edquist, 1996, Robinson et al., 2007).

It has been suggested that abnormal luteal function is due to inadequate follicular development before ovulation (Southee et al., 1988b, McLeod et al., 1982b) and inadequate development of the microvasculature of the pre-ovulatory follicle and subsequent CL (Southee et al., 1988a).

Multiple small-dose injections of GnRH has been shown to successfully induce ovulation in seasonally anoestrous ewes (Southee et al., 1988b, McLeod et al., 1983, McLeod et al., 1982a). However, unless primed with progesterone prior to GnRH treatment, the vast majority of animals develop a defective CL (Haresign et al., 1996, Husein and Kriddli, 2003, Hunter et al., 1988). This provides us with a reliable *in vivo* model to study the differences in late follicular and early luteal development of functional and defective CL.

One working hypothesis is that the lack of progesterone priming prior to GnRH-induced ovulation results in the abnormal production of angiogenic factors

during late follicular development and early luteal formation. Defective production of these factors could lead to the formation of an inadequate vascular bed and limited development of the dominant follicle prior to the LH surge. This deficient blood supply to the dominant follicle could lead to the formation of a poor and unstable vascular network, during early luteal development, incapable of supporting the newly formed CL and thus resulting in defective luteal function. Furthermore, abnormal production of angiogenic factors after the LH surge could result in restricted sprouting of blood vessels from the theca into the granulosa layer after the basal membrane dissolves. This could further interfere with the luteinisation process taking place as well as compromising angiogenesis during the early luteal phase.

The current experiment was designed to test this hypothesis and investigate the effects of progesterone priming on the expression of angiogenic factors in ovine follicles, both before and after the LH surge.

3.2 Material and Methods

Seasonally anoestrous ewes were allocated into four treatment groups (Groups 1 to 4) of which two received an i.m. injection of 20 mg of progesterone 3 days prior to the start of GnRH treatment (Groups 2 and 4) while the remaining received oil vehicle alone (Groups 1 and 3). Small-dose GnRH injections were given every 2 hours for 28 hours followed by a bolus injection at 30 hours, as described in detail in Chapter 2.

In order to determine angiogenic factors levels in the follicular cell layers prior to the LH surge, ovaries from Groups 1 and 2 were collected 24 hours after the start of GnRH treatment (6 hours prior to GnRH bolus injection and expected pre-ovulatory LH surge). In order to analyse angiogenic factor levels post-LH surge, ovaries from Groups 3 and 4 were collected 46 hours after the start of GnRH treatment (16 hours after GnRH bolus injection and approximately 14 hours prior to expected ovulation). Animals that happened to ovulate (1/10 from progesterone primed and 2/10 from non-primed animals) were excluded from any analysis.

Blood samples were taken every 2 hours to monitor the timing of the pre-ovulatory LH surge. The samples were collected just prior to GnRH injections, from the start of GnRH treatment until 6 hours before (Groups 1 and 2) and 16 hours after (Group 3 and 4) GnRH bolus injection. For a period of 4 hours, at the beginning of GnRH treatment, samples were collected more frequently to monitor if GnRH injections were inducing the desired LH pulses.

3.2.1 LH ELISA assays

In order to confirm the onset of the LH surge, LH ELISA immunoassays were performed on plasma samples as described in Chapter 2.

3.2.2 Immunohistochemistry

Immunohistochemistry assays were performed according to the process described in Chapter 2. However, optimized primary antibody concentration and colour developing time varied between proteins and are detailed below.

For VEGF, slides were incubated in a 1:100 concentration (1 µl of the antibody in 99 µl of PBS) of Rabbit polyclonal anti-human VEGF antibody at 200 µg/ml (VEGFA-20, Santa Cruz Biotechnologies) overnight for 23 hours and then left in contact with DAB solution to develop colour for 1 minute.

Rabbit polyclonal anti-mouse FLK-1 (VEGFR-2) at 200 µg/ml (Santa Cruz Biotechnologies) was used overnight for 24 hours at a concentration of 1:50. Slides were left to develop colour for 2 minutes.

For Ang-1, a 1:200 concentration of Rabbit polyclonal anti-mouse Angiopoietin-1 at 85 mg/ml (Abcam) was used for 1 hour and slides were left to develop colour for 2 minutes.

For Ang-2 the antibody concentration used was of 1:100 Rabbit polyclonal anti-human Angiopoietin-2 at 200 µg/ml (Abcam) for 1 hour and colour development of 2 minutes.

For TIE-2, 1:100 Rabbit polyclonal anti-mouse TIE-2 at 200 μg/ml (C-20, Santa Cruz Biotechnologies) was used for 1 hour and slides were left in contact with DAB for 30 seconds to develop colour.

3.2.3 *In situ* hybridization

For every gene of interest there were 294 positive (AS) and 98 negative (S) sections. Enough probe was made to cover that number with a safe margin and also to be able to repeat half of the assay if needed. Due to the large number of slides involved, each mRNA of interest had slides divided into 2 assays. Each assay contained slides from animals belonging to each group, in equal numbers. *In situ* hybridization was performed according to the technique described in Chapter 2. Optimized hybridization and colour development for each mRNA of interest are described below.

3.2.3.1 Angiopoietins and their receptor

Ang-1, Ang-2 and TIE-2 hybridization was performed for 19 hours in a humidified chamber at 60 °C. Colour development took place for 20 hours prior to mounting.

3.2.3.2 VEGF and VEGFR-2

Professor Dale Redmer (Department of Animal and Range Sciences, North Dakota State University, Fargo, ND, USA) kindly provided bacteria containing VEGF and VEGFR-2 cDNA as described in Redmer *et al.* (1996). Bacteria were replicated and plasmid successfully extracted.

VEGFR-2 cDNA linearization proved successful and although S and AS probes obtained were extremely low in concentration, after many attempts, there was enough probe for CL slides but not enough for follicles. VEGF cDNA linearization was not successful and therefore riboprobes for VEGF could not be made.

3.2.4. Statistical analysis

Prior to the analysis follicles were classified into small (2 – 2.5 mm in diameter) and large (>2.5 mm in diameter) and analysed separately. For each analysis of a factor there was a total of approximately 91 small and 79 large follicles.

The data on plasma LH concentration were analyzed using One-way ANOVA. Both protein and mRNA Expression Indices for different angiogenic factors were analyzed using a Linear Mixed Model within the REML function of GenStat using Treatment x Time x Layer as fixed effects and Animal/Follicle number as random effects. If data were not normally distributed the best power of transformation to normalise the residuals was identified using the Lambda value with maximum likelihood. Values were submitted to Log (Ang-1 protein in small follicles and VEGF protein in large follicles) or Square Root transformation (Ang-1 and -2 mRNA in small follicles and Ang-1 mRNA in large follicles). Due to the fact that some of the values equaled to zero, Log of Value+1 was used to make the conversion possible. For ease of interpretation back transformed mean values are provided in the tables. Overall effects of treatment (progesterone priming), time (before or after the LH surge) and layer (granulosa, theca interna and theca externa) as well as 3-way interactions (treatment x time x layer) were analyzed. All analyses were performed using GenStat 9th edition (Version 9.1.0.147, Lawes Agricultural Trust, VSN International Ltd). Significance was considered at 5% level.

3.3 Results

3.3.1 LH profile

Mean plasma LH concentrations in the different experimental groups are illustrated in Figure 19.

Mean LH concentrations did not vary between treatment groups at any time of sample collection. LH levels remained low prior to GnRH bolus. However, from 6 to 10 hours after the start of pulsatile GnRH treatment when animals were sampled more frequently and soon after rather than immediately prior to GnRH injections, LH

concentrations fluctuated and were comparatively higher indicating that each GnRH injection was inducing a LH pulse. Values, however, did not exceed 3 ng/ml prior to GnRH bolus injection. The GnRH bolus, that was given intravenously 30 hours after the beginning of the pulsatile GnRH treatment, successfully induced an immediate LH surge in all animals.

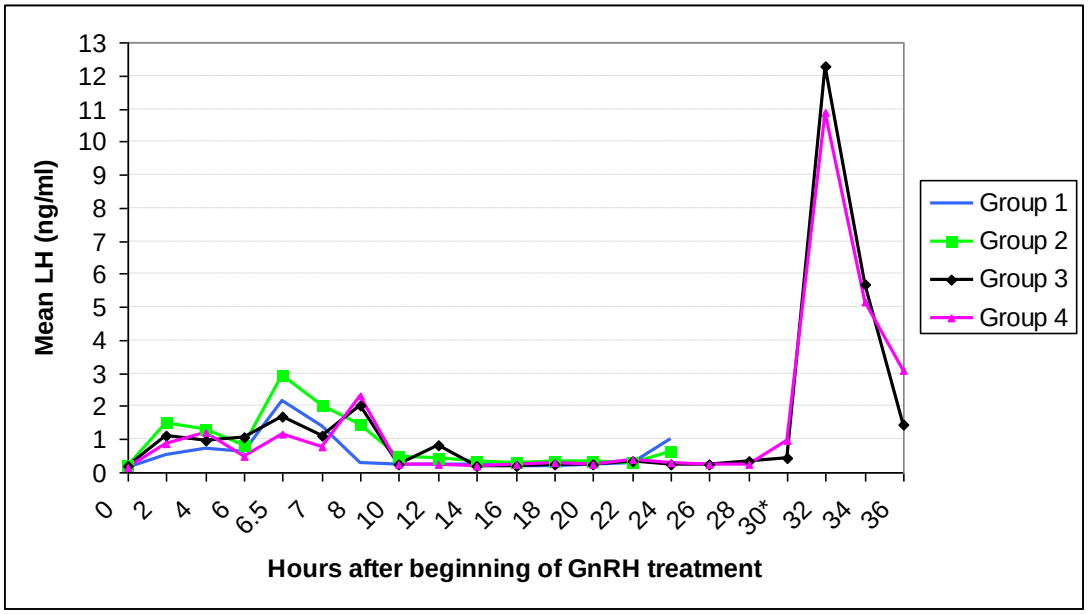


Figure 19. Mean plasma LH concentrations of progesterone primed (Groups 2 and 4) and non-primed (Groups 1 and 3) seasonally anoestrous ewes at various times after start of GnRH treatment. GnRH bolus injection (300 µg) was given intravenously at 30 hours (*) after the start of pulsatile GnRH treatment.

3.3.2 Small follicles

3.3.2.1 VEGF protein

An example of VEGF protein expression in the various follicular layers can be seen in Figure 20. Statistical analysis indicated that there was no significant effect (P = 0.69) of progesterone priming on VEGF protein levels. However, there were significant effects (P<0.05) of time of LH surge and follicular cell layers on VEGF protein levels but no significant interaction (P = 0.40) between progesterone priming, time and follicular layers.

Overall mean VEGF protein expression was significantly higher ($P<0.05$) before compared with that after the LH surge (Figure 21). The expression in the theca interna layer was significantly higher ($P<0.05$) than in the two other layers, with no significant difference between the theca externa and granulosa layers (Figure 22, Table 4).

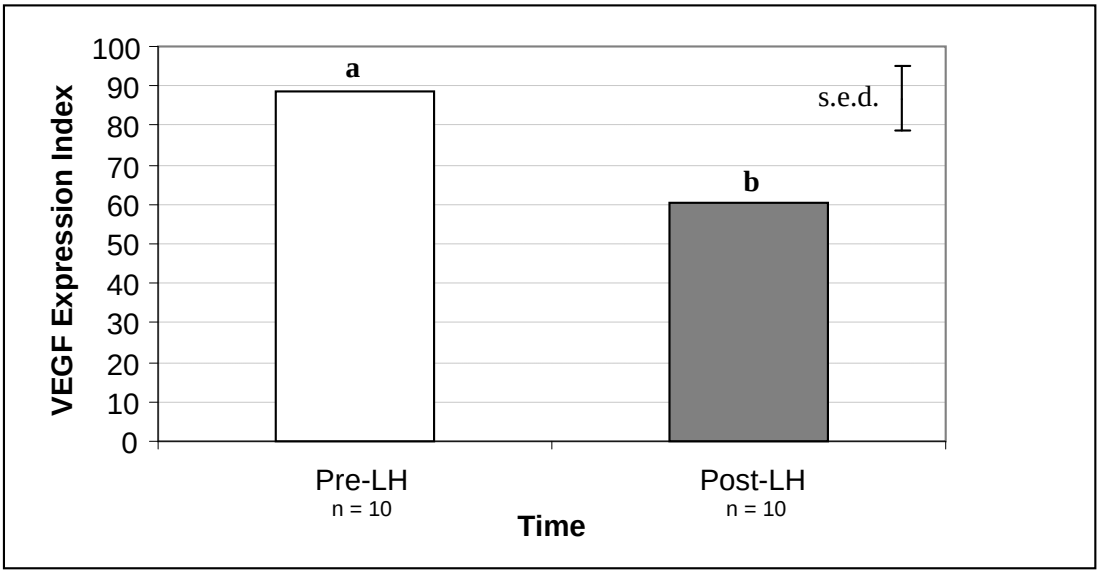


Figure 21. The overall mean Expression Index of VEGF protein before and after the pre-ovulatory LH surge in small follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 8.12, standard error of difference between means to compare the mean values for VEGF expression before and after the LH surge. Different letters on bars indicate significant difference at 5% level. n = number of animals.

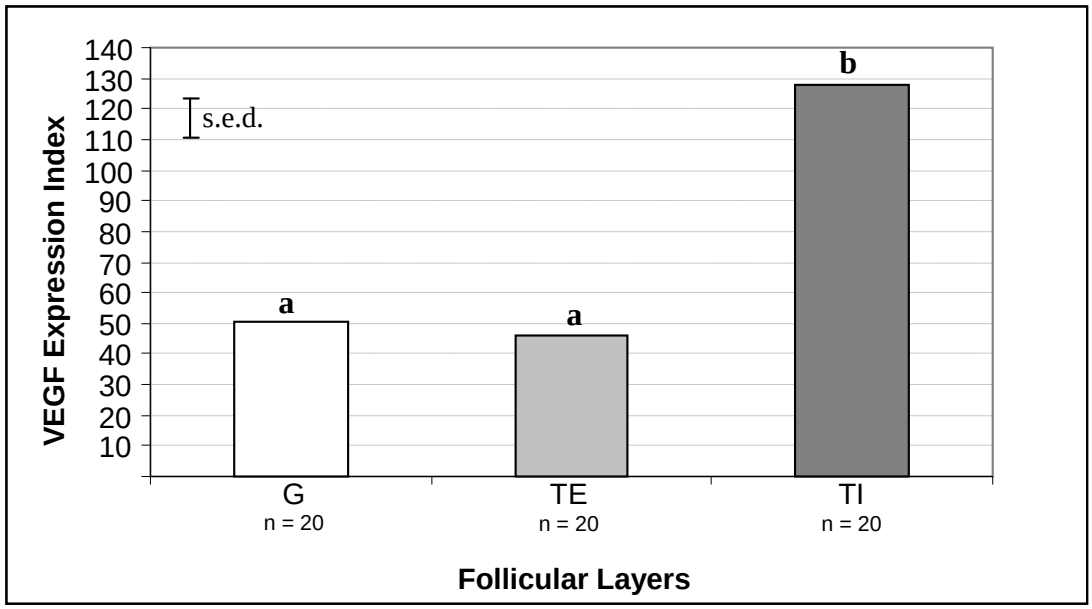


Figure 22. The overall mean Expression Index of VEGF protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 6.355, standard error of difference between means to compare the mean values for VEGF expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 4. Mean VEGF protein Expression Index values observed in the various cell layers of small follicles of the different groups of progesterone primed and non-primed ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	55.04	46.60	144.59
2	+	Pre-LH	81.68	58.78	144.82
3	-	Post-LH	22.71	28.76	97.02
4	+	Post-LH	41.76	49.21	123.79

s.e.d. (14.97) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

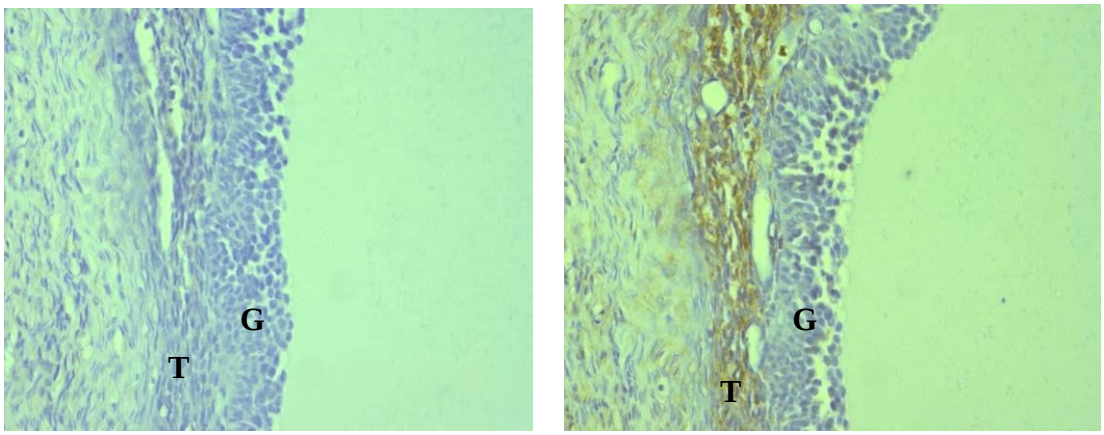


Figure 20. Immunohistochemistry showing follicular VEGF protein expression, after the LH surge, in the ovary of a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH without progesterone priming. Negative (left) and positive (right) staining for VEGF protein is shown. The granulosa (G) and the theca (T) layers are identified. Bright field microscopy, 200X magnification.

3.3.2.2 VEGFR-2 protein

An example of VEGFR-2 protein expression in the various follicular layers can be seen in Figure 23. Statistical analysis indicated that there was no significant effect of progesterone priming ($P = 0.34$) nor timing of the LH surge ($P = 0.93$) on VEGFR-2 protein levels. However, there was a significant effect ($P<0.05$) of follicular cell layers on VEGFR-2 protein levels but no significant interaction ($P = 0.18$) between factors.

The theca interna layer of small follicles expressed significantly higher ($P<0.05$) VEGFR-2 protein levels than the other two layers. Furthermore, VEGFR-2 protein expression was significantly lower ($P<0.05$) in the granulosa than in the theca externa layer (Figure 24).

Mean VEGFR-2 protein values, observed in the different cell layers of small follicles of progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, before and after the LH surge are detailed in Table 5.

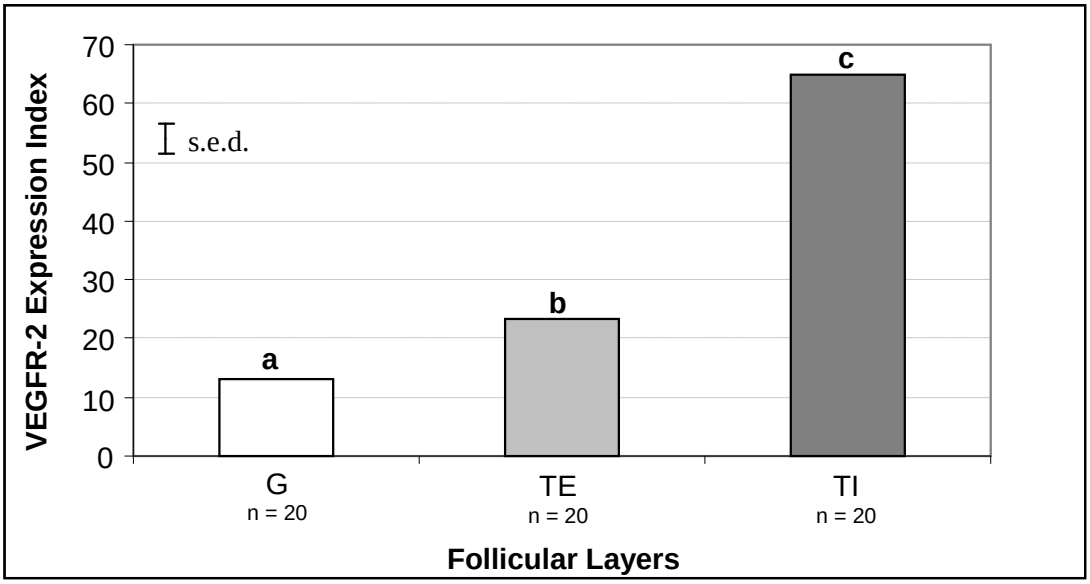


Figure 24. The overall mean Expression Index of VEGFR-2 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 2.516, standard error of difference between means to compare the mean values for VEGFR-2 expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 5. Mean VEGFR-2 protein Expression Index values observed in the various cell layers of small follicles of the different groups of progesterone primed and non-primed ewes treated with GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	11.77	23.29	69.83
2	+	Pre-LH	12.91	23.92	64.72
3	-	Post-LH	7.36	13.97	50.01
4	+	Post-LH	20.46	31.75	74.90

s.e.d. (10.07) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

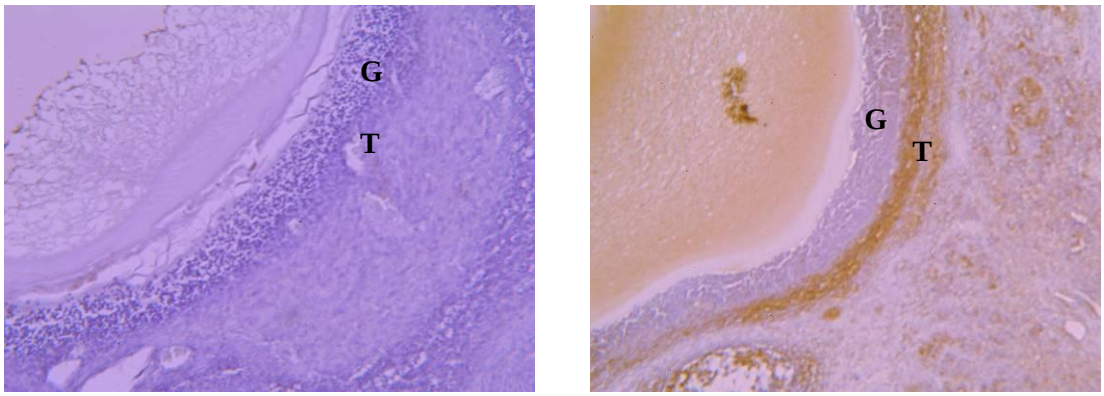


Figure 23. Immunohistochemistry showing follicular VEGFR-2 protein expression, after the LH surge, in the ovary of a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH primed with progesterone. Negative (left) and positive (right) staining for VEGFR-2 protein is shown. The granulosa (G) and theca (T) layers are identified. Bright field microscopy, 100X magnification.

3.3.2.3 Angiopoietin-1 protein

An example of Ang-1 protein expression in the various follicular layers can be seen in Figure 25. As data were not normally distributed, values were submitted to Log transformation prior to statistical analysis. There was no significant effect of progesterone priming ($P = 0.32$) nor timing of the LH surge ($P = 0.73$) on Ang-1 protein expression. However, there were significant effects ($P<0.05$) of follicular cell layers on Ang-1 protein levels as well as a significant interaction between factors.

The theca interna layer expressed significantly higher ($P<0.05$) amounts of Ang-1 protein than the granulosa and theca externa layers. The theca externa expressed significantly higher ($P<0.05$) levels of Ang-1 protein than the granulosa layer (Figure 26).

Mean Ang-1 protein values observed in the different cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 6 and illustrated in Figure 27. Although statistical analysis indicated that there was a significant ($P<0.05$) interaction between the factors there was no consistent pattern evident in these data to account for this significance.

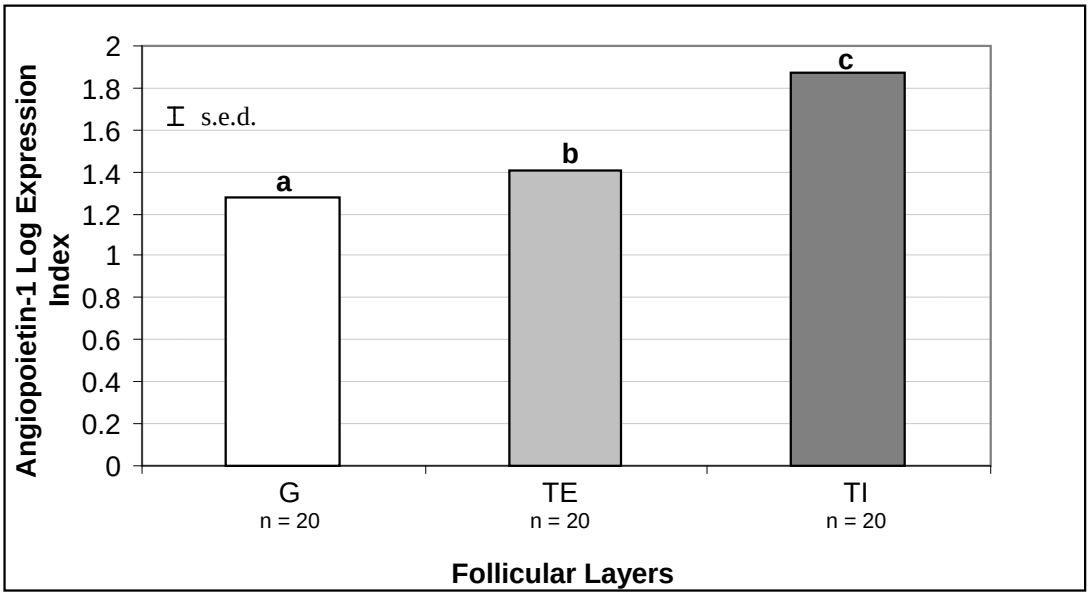


Figure 26. The overall mean Log Expression Index of Angiopoietin-1 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. Log s.e.d. = 0.039, standard error of difference between means to compare the mean values for Angiopoietin-1 expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 6. Back transformed mean values for Angiopoietin-1 protein Expression Index (Log values) observed in the various cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	16.55 (1.219)	25.76 (1.411)	88.30 (1.946)
2	+	Pre-LH	25.35 (1.404)	30.47 (1.484)	67.92 (1.832)
3	-	Post-LH	14.15 (1.151)	13.96 (1.145)	52.48 (1.720)
4	+	Post-LH	22.23 (1.347)	37.58 (1.575)	94.84 (1.977)

Log s.e.d. (0.2244) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

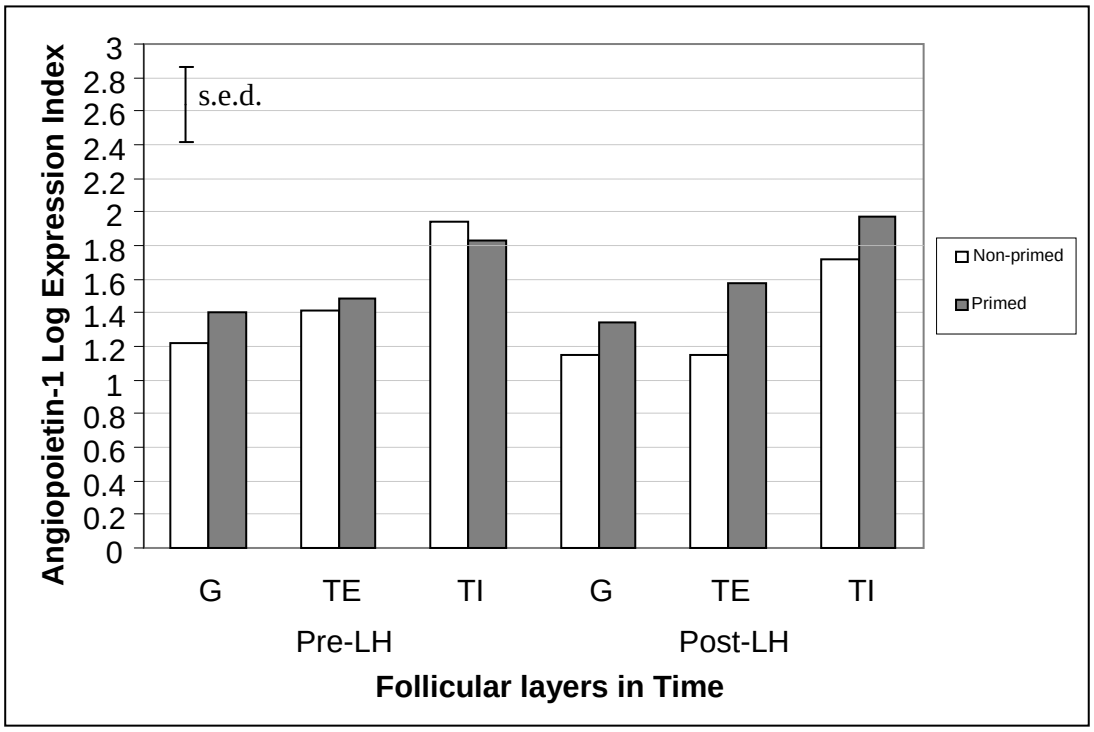


Figure 27. The mean Log Expression Index of Angiopoietin-1 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, prior to and post-LH surge. The Log s.e.d. = 0.2244, standard error of the difference between means to compare mean values within and between treatment groups. Number of animals per treatment per time = 5.

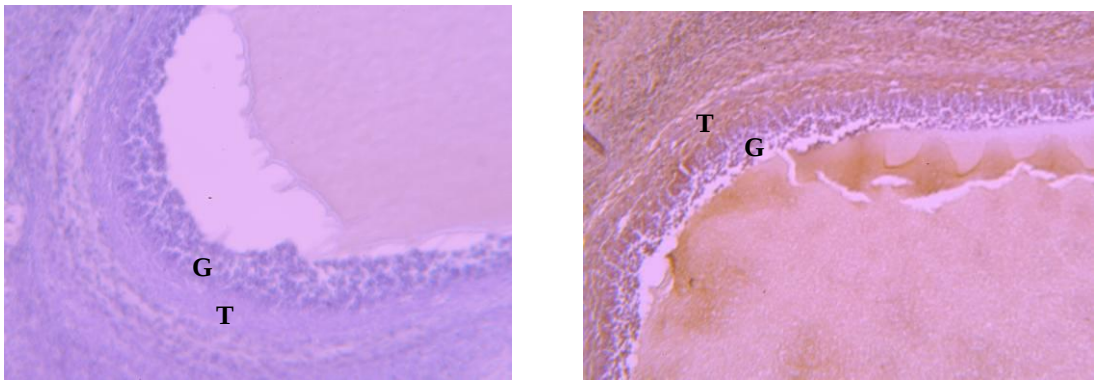


Figure 25. Immunohistochemistry showing follicular Angiopoietin-1 protein expression, after the LH surge, in the ovary of a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH primed with progesterone. Negative (left) and positive (right) staining for Angiopoietin-1 protein is shown. The granulosa (G) and theca (T) layers are identified. Bright field microscopy, 100X magnification.

3.3.2.4 Angiopoietin-1 mRNA

An example of Ang-1 mRNA expression in the various follicular layers can be seen in Figure 28. As data were not normally distributed, values were submitted to Square Root transformation prior to statistical analysis. There was no significant effect of progesterone priming ($P = 0.98$) nor timing of the LH surge ($P = 0.62$) on Ang-1 mRNA expression levels. However, there was a significant effects ($P<0.05$) of follicular cell layers on Ang-1 mRNA expression levels with no significant interaction between factors ($P = 0.27$).

The granulosa showed significantly higher ($P<0.05$) Ang-1 mRNA expression levels than both theca layers. The expression in theca interna was significantly higher ($P<0.05$) than in the theca externa layer (Figure 29).

Mean Ang-1 mRNA values observed in the different cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 7.

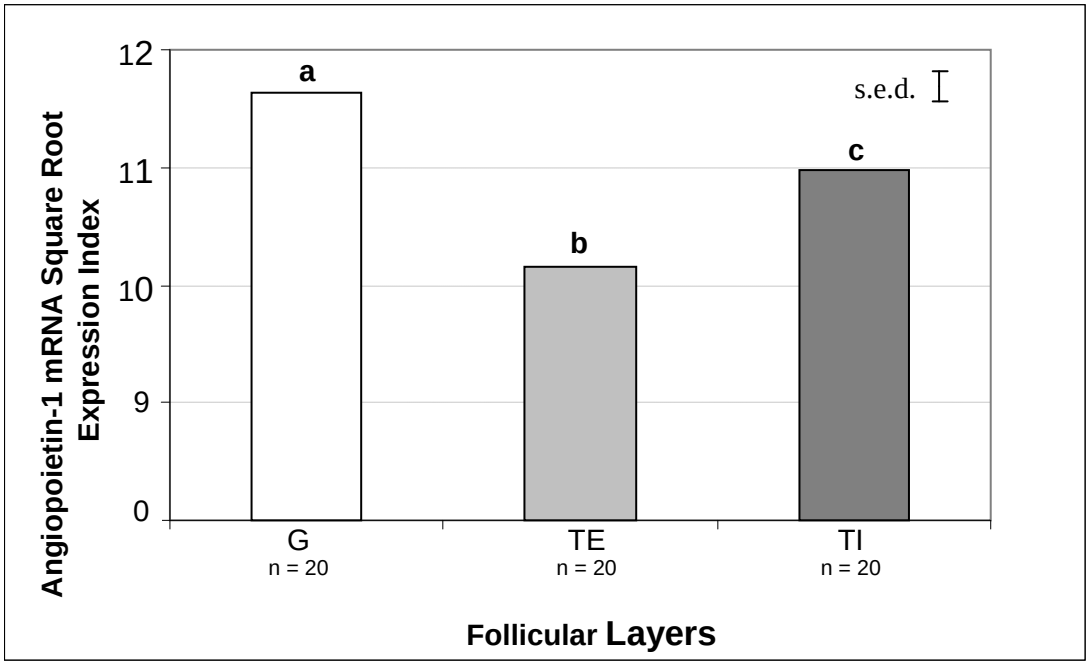


Figure 29. The overall mean Square Root Expression Index of Angiopoietin-1 mRNA in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. Square Root s.e.d.=0.1259, standard error of difference between means to compare the mean values for Angiopoietin-1 expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

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Table 7. Back transformed mean values of Angiopoietin-1 mRNA Expression Index (Square Root values) observed in the various cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	145.44 (12.06)	105.47 (10.27)	115.56 (10.75)
2	+	Pre-LH	131.10 (11.45)	103.22 (10.16)	120.34 (10.97)
3	-	Post-LH	130.41 (11.42)	102.41 (10.12)	118.81 (10.90)
4	+	Post-LH	136.56 (11.60)	100.80 (10.04)	127.46 (11.29)

Square root s.e.d. (0.325) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

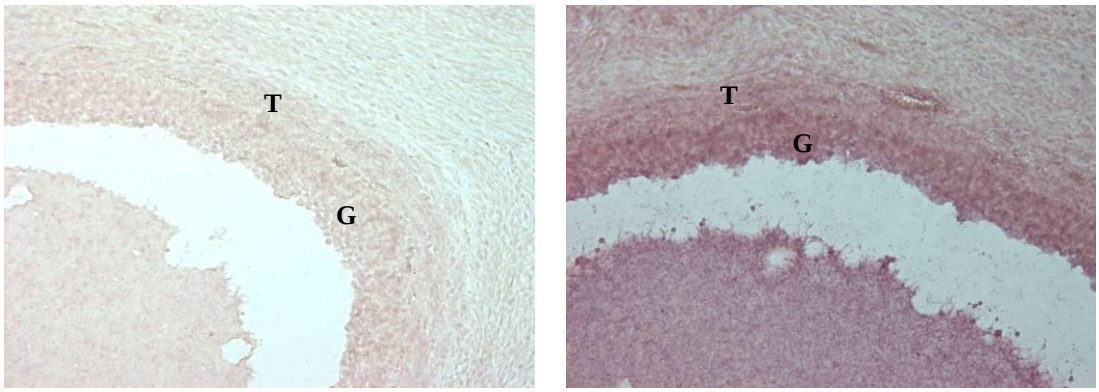

Figure 28. *In situ* hybridization showing follicular Angiopoietin-1 mRNA expression, after the LH surge, in the ovary of a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH without progesterone priming. Sense (left) and anti-sense (right) probed slides for Angiopoietin-1 mRNA are shown. The granulosa (G) and theca (T) layers are shown. Bright field microscopy, 200X magnification.

3.3.2.5 Angiopoietin-2 protein

An example of Ang-2 protein expression in the various follicular layers can be seen in Figure 30. Statistical analysis indicated that there was no significant effect of progesterone priming ($P = 0.54$) nor timing of the LH surge ($P = 0.60$) on Ang-2 expression levels. However, there were significant effects ($P<0.05$) of follicular cell layers on Ang-2 protein levels as well as of the interaction between factors.

Significant difference ($P<0.05$) in Ang-2 protein levels were observed between all three follicular layers, with the highest values in the theca interna and the lowest levels in the granulosa layer (Figure 31).

Mean Ang-2 protein values observed in the different cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 8.

It appears as though the significant interaction was due to the lower levels of Ang-2 protein in progesterone primed and non-primed animals before the LH surge and high levels after the LH surge, and this was consistent in all follicular layers (Figure 32).

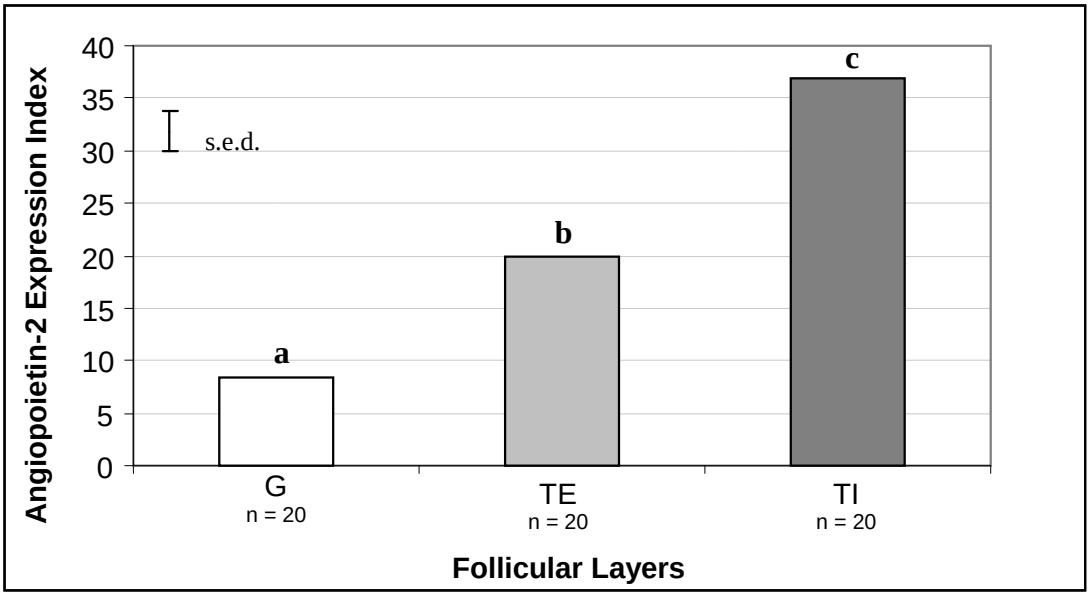

Figure 31. The overall mean Expression Index of Angiopoietin-2 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 1.910, standard error of difference between means to compare the mean values for Angiopoietin-2 expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 8. Mean Angiopoietin-2 protein Expression Index values observed in the various cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	12.62	22.36	45.46
2	+	Pre-LH	6.43	18.44	27.98
3	-	Post-LH	3.49	15.68	31.47
4	+	Post-LH	11.28	23.36	42.77

Square root s.e.d. (9.92) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

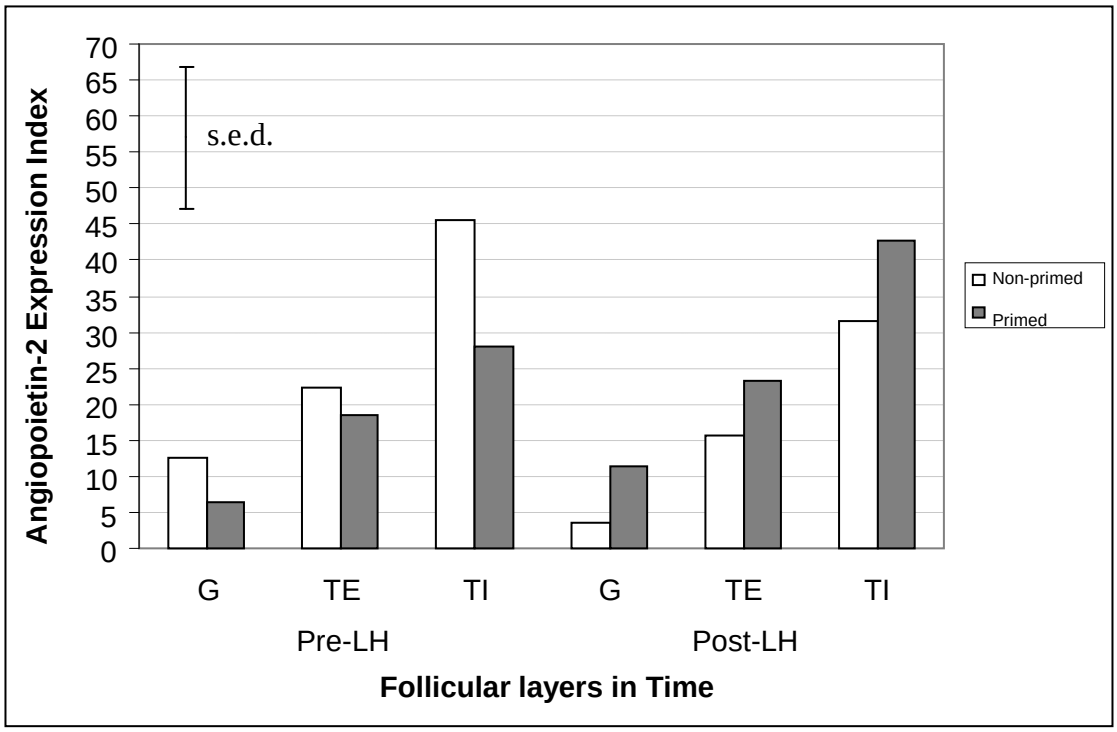

Figure 32. The mean Expression Index of Angiopoietin-2 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, prior to and post-LH surge. The s.e.d. = 9.925, standard error of the difference between means to compare mean values within and between treatment groups. Number of animals per treatment per time = 5.

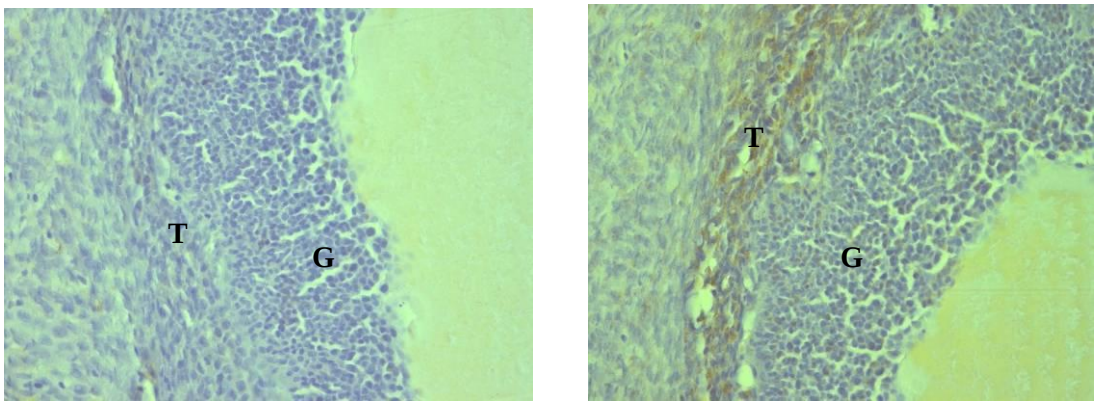

Figure 30. Immunohistochemistry showing follicular Angiopoietin-2 protein expression, before the LH surge, in the ovary of a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH primed with progesterone. Negative (left) and positive (right) staining for Angiopoietin-2 protein is shown. The granulosa (G) and theca (T) layers are identified. Bright field microscopy, 400X magnification.

3.3.2.6 Angiopoietin-2 mRNA

An example of Ang-2 mRNA expression in the various follicular layers can be seen in Figure 33. As data was not normally distributed, values were submitted to Square Root transformation prior to analysis. There was no significant effect of progesterone priming ($P = 0.54$) nor timing of the LH surge ($P = 0.82$) on Ang-2 mRNA expression levels. However, there was a significant effect ($P<0.05$) of follicular cell layers on Ang-2 mRNA expression levels with no significant interaction between factors ($P = 0.99$).

The granulosa layer showed small but significant higher ($P<0.05$) levels of Ang-2 mRNA expression than the theca layers, with no significant difference being observed between the theca externa and theca interna layers (Figure 34).

Mean Ang-2 mRNA values observed in the different cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 9.

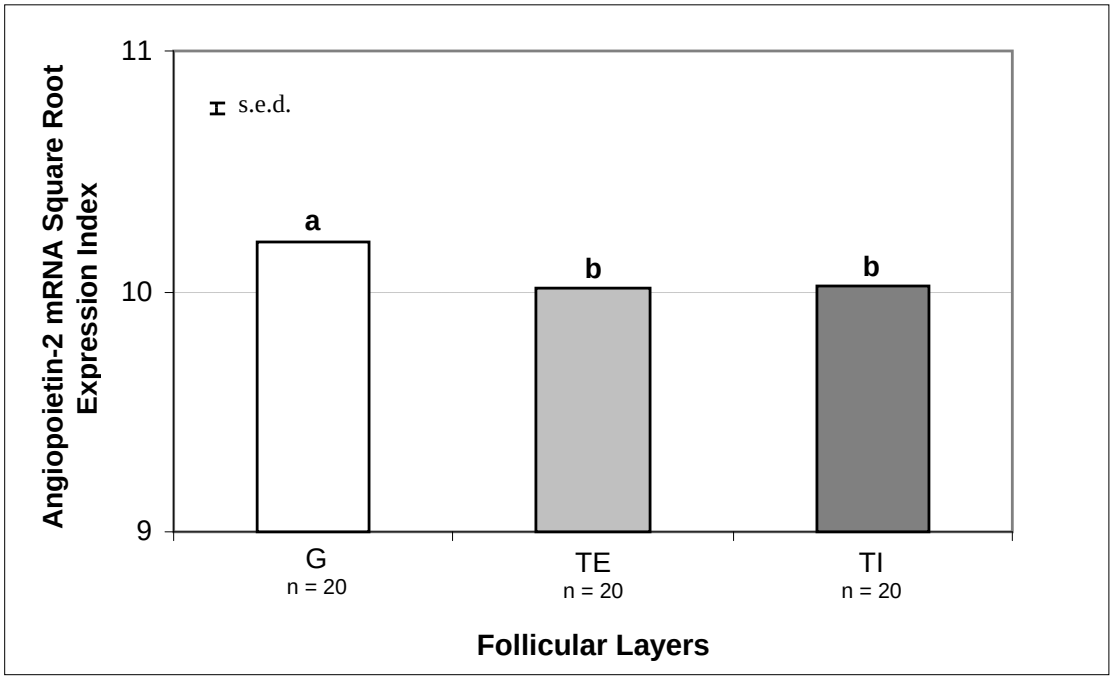

Figure 34. The overall mean Square Root Expression Index of Angiopoietin-2 mRNA in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. Square Root s.e.d. = 0.024, standard error of difference between means to compare the mean values for Angiopoietin-2 mRNA expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 9. Back transformed mean values of Angiopoietin-2 mRNA Expression Index (Square Root values) observed in the various cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	105.06 (10.25)	100.00 (10.00)	100.60 (10.03)
2	+	Pre-LH	103.02 (10.15)	100.60 (10.03)	101.20 (10.06)
3	-	Post-LH	105.88 (10.29)	100.00 (10.00)	100.00 (10.00)
4	+	Post-LH	103.02 (10.15)	100.00 (10.00)	100.00 (10.00)

Square root s.e.d. (0.068) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

Figure 33. *In situ* hybridization showing follicular Angiopoietin-2 mRNA expression, after the LH surge, in the ovary of a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH primed with progesterone. Sense (left) and anti-sense (right) probed slides for Angiopoietin-2 mRNA are shown. The granulosa (G) and theca (T) layers are identified. Bright field microscopy, 200X magnification.

3.3.2.7 TIE-2 protein

An example of TIE-2 protein expression in the various follicular layers can be seen in Figure 35. Statistical analysis indicated that there was no significant effect of progesterone priming (P = 0.29) nor timing (P = 0.24) on TIE-2 protein levels. There were no significant effects (P = 0.12) of follicular cell layers on Ang-2 protein levels nor of the interaction between factors (P = 0.15).

Mean TIE-2 protein values observed in the different cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 10.

Table 10. Mean TIE-2 protein Expression Index values observed in the various cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	13.02	15.67	18.26
2	+	Pre-LH	10.46	12.40	10.15
3	-	Post-LH	6.82	9.39	6.89
4	+	Post-LH	5.59	4.88	5.51

s.e.d. (6.84) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

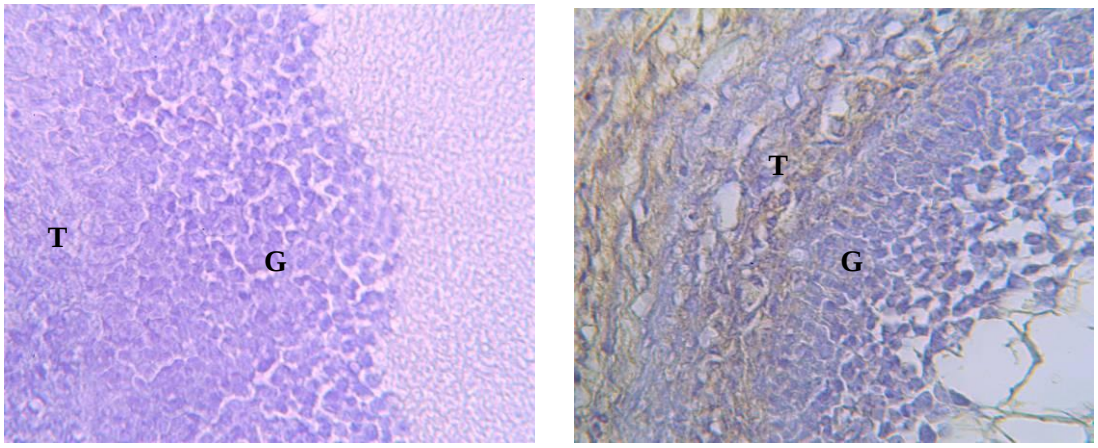

Figure 35. Immunohistochemistry showing follicular TIE-2 protein expression, after the LH surge, in the ovary of a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH primed with progesterone. Negative (left) and positive (right) staining for TIE-2 protein is shown. The granulosa (G) and theca (T) layers are identified. Bright field microscopy, 400X magnification.

3.3.2.8 TIE-2 mRNA

An example of TIE-2 mRNA expression in the various follicular layers can be seen in Figure 36. Statistical analysis indicated that there was no significant effect of progesterone priming (P = 0.19) nor timing of the LH surge (P = 0.87) on TIE-2 mRNA expression levels. However, there was a significant effect (P<0.05) of follicular cell layers on Ang-2 mRNA expression levels with no significant interaction between factors (P = 0.37).

The granulosa expressed significantly higher (P<0.05) levels of TIE-2 mRNA than the theca layers, with no significant differences being observed between the theca layers (Figure 37).

Mean TIE-2 mRNA values observed in the different cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 11.

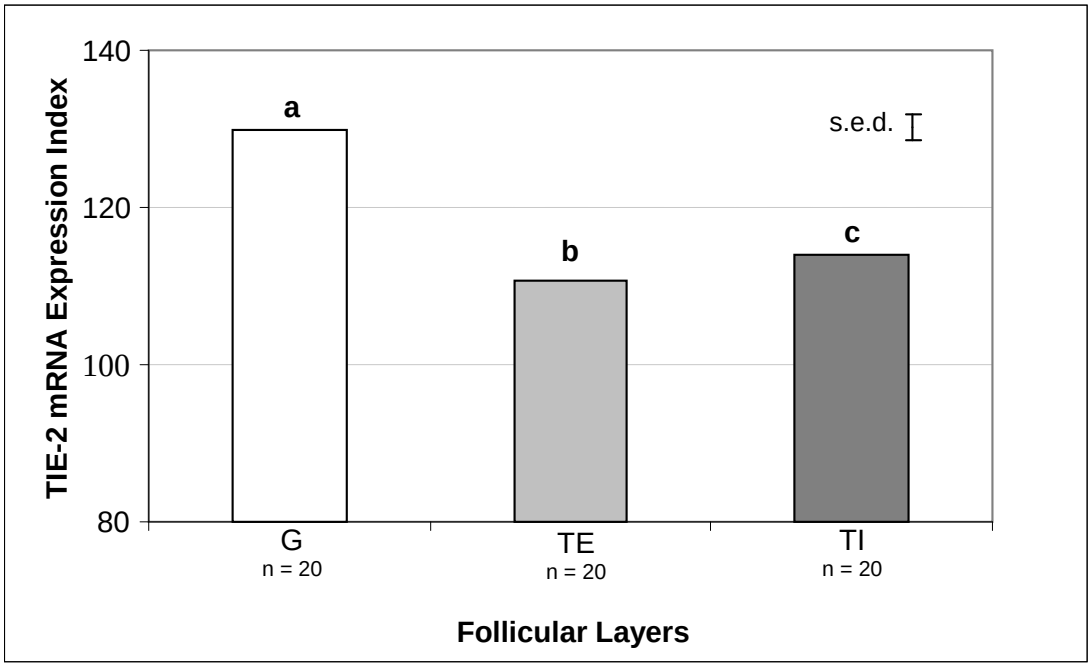

Figure 37. The overall mean Expression Index of TIE-2 mRNA in the granulosa (G), theca externa (TE) and theca interna (TI) layers of small follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 1.685, standard error of difference between means to compare the mean values for TIE-2 mRNA expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 11. Mean TIE-2 mRNA Expression Index values observed in the various cell layers of small follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	121.4	103.3	103.1
2	+	Pre-LH	139.3	123.5	124.1
3	-	Post-LH	123.6	104.6	111.2
4	+	Post-LH	135.6	111.9	117.2

s.e.d. (12.83) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

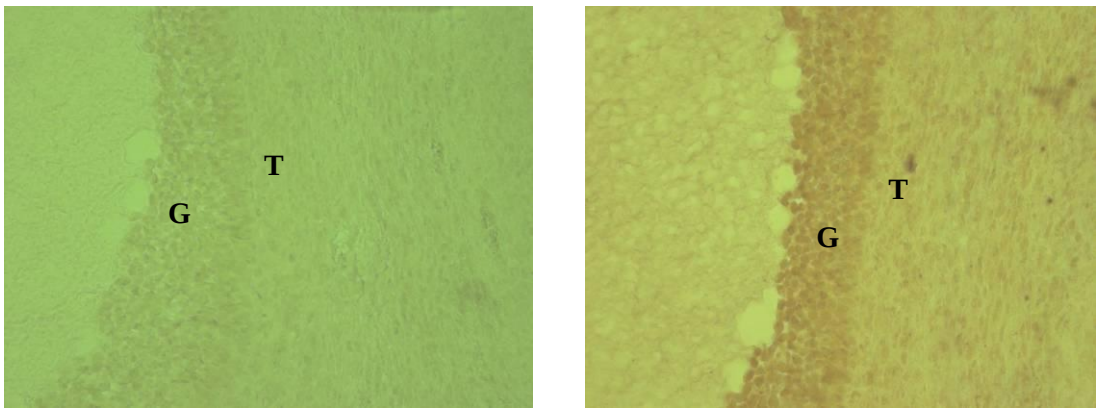

Figure 36. *In situ* hybridization showing follicular TIE-2 mRNA expression, after the LH surge, in the ovary of a seasonally anoestrous ewe treated with small-dose multiple injections of GnRH without progesterone priming. Sense (left) and anti-sense (right) probed slides for TIE-2 mRNA are shown. The granulosa (G) and theca (T) layers are identified. Bright field microscopy, 200X magnification.

3.3.3 Large follicles

3.3.3.1 VEGF protein

As data was not normally distributed, values were submitted to Log transformation prior to analysis. Statistical analysis indicated that there was a significant effect ($P<0.05$) of progesterone priming, timing of the LH surge and follicular cell layers on VEGF protein expression but no significant interaction ($P = 0.98$) between progesterone priming, time and follicular layers.

Progesterone primed animals showed significantly higher ($P<0.05$) overall mean levels of VEGF protein than non-primed animals (Figure 38).

Overall mean VEGF protein expression levels were found to be significantly higher ($P<0.05$) prior to the LH surge (Figure 39).

Overall mean VEGF protein expression levels were significantly higher ($P<0.05$) in the theca interna layer than in the remaining two layers with no significant difference being observed between the granulosa and theca externa layers (Figure 40).

Mean VEGF protein expression values observed in the different cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with GnRH are detailed in Table 12.

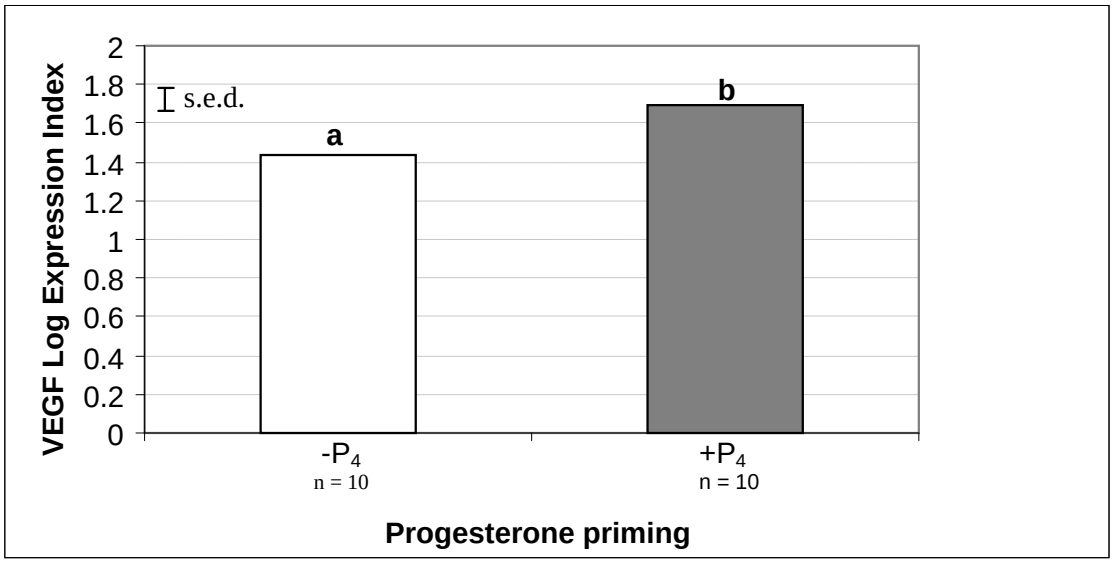

Figure 38. The overall mean Log Expression Index of VEGF protein in large follicles of progesterone primed (+P4) and non-primed (-P4) seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. Log s.e.d. = 0.058, standard error of difference between means to compare the mean values for VEGF expression before and after the LH surge. Different letters on bars indicate significant difference at 5% level. n = number of animals.

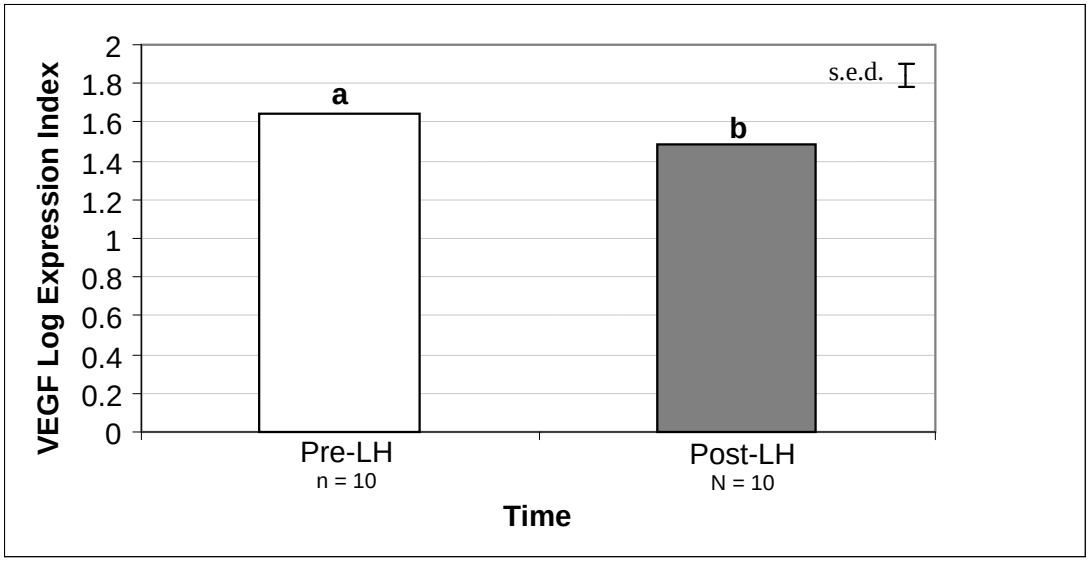

Figure 39. The overall mean Log Expression Index of VEGF protein before and after the pre-ovulatory LH surge in large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. Log s.e.d. = 0.058, standard error of difference between means to compare the mean values for VEGF expression before and after the LH surge. Different letters on bars indicate significant difference at 5% level. n = number of animals.

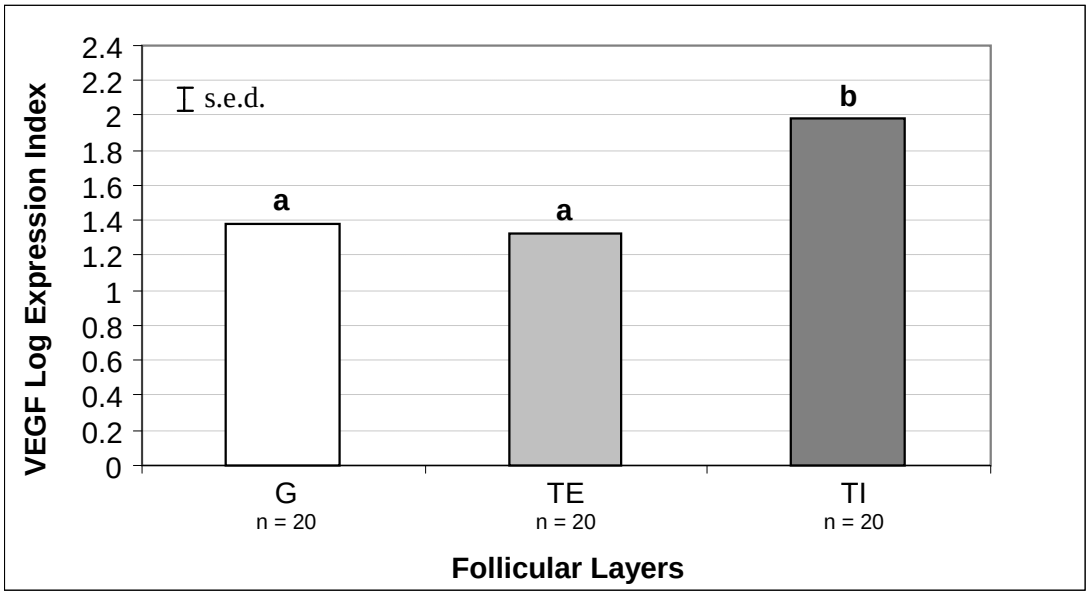

Figure 40. The overall mean Log Expression Index of VEGF protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. Log s.e.d. = 0.066, standard error of difference between means to compare the mean values for VEGF expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 12. Back transformed mean values for VEGF protein Expression Index (Log values) observed in the various cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	23.83 (1.377)	20.79 (1.318)	92.57 (1.965)
2	+	Pre-LH	53.70 (1.730)	25.40 (1.405)	117.76 (2.071)
3	-	Post-LH	8.79/ (0.944)	14.89 (1.173)	69.02 (1.839)
4	+	Post-LH	29.17 (1.465)	25.40 (1.405)	118.30 (2.073)

Log s.e.d. (0.136) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

3.3.3.2 VEGFR-2 protein

Statistical analysis indicated that there was no significant effect of progesterone priming ($P = 0.14$) nor timing of the LH surge ($P = 0.55$) on VEGFR-2 protein expression. However, there were significant effects ($P<0.05$) of follicular cell layers on VEGFR-2 protein expression as well as significant interaction between factors.

The theca interna expressed significantly higher ($P<0.05$) amounts of VEGFR-2 protein than the granulosa and theca externa layers, with no significant difference being observed between the granulosa and theca externa layers (Figure 41).

No significant differences in VEGFR-2 protein expression levels could be observed in the granulosa and theca externa layers between progesterone primed and non-primed animals either prior to or after the LH surge. However, prior to the LH surge, non-primed animals expressed significantly higher levels of VEGFR-2 protein in the theca interna than primed animals, but after the LH surge progesterone primed animals expressed significantly higher levels of VEGFR-2 expression in the theca interna layer than non-primed animals (Figure 42). It appears as though the significant interaction was due to lower VEGFR-2 expression levels in the theca interna of primed animals before the LH surge, but higher levels after the LH.

Mean VEGFR-2 protein expression values observed in the different cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 13.

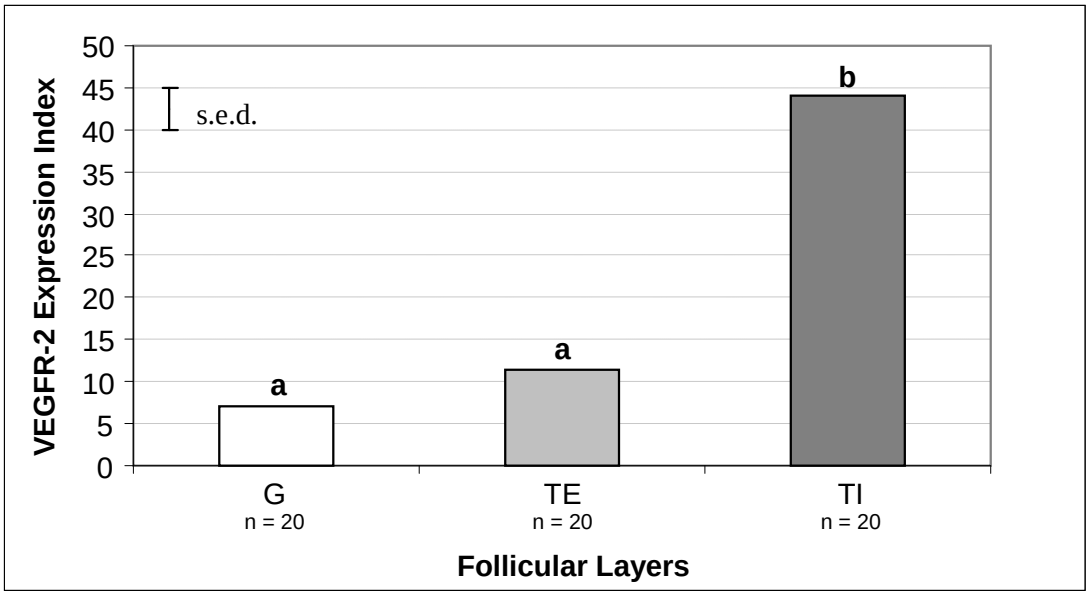

Figure 41. The overall mean Expression Index of VEGFR-2 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 2.55, standard error of difference between means to compare the mean values for VEGFR-2 expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 13. Mean VEGFR-2 protein Expression Index values observed in the various cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	6.73	9.02	48.32
2	+	Pre-LH	9.76	11.38	31.70
3	-	Post-LH	3.93	7.05	31.46
4	+	Post-LH	7.92	18.39	65.18

s.e.d. (8.22) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

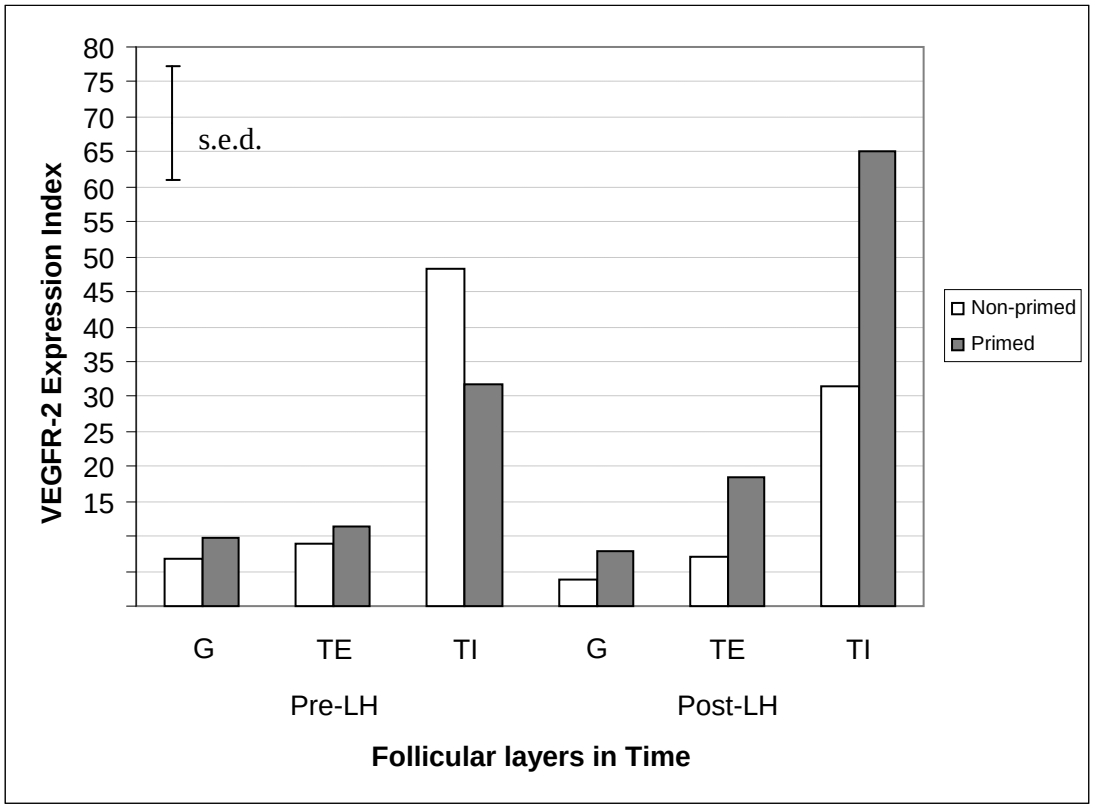

Figure 42. The mean Expression Index of VEGFR-2 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, prior and post-LH surge. The s.e.d. = 8.227, standard error of the difference between means to compare mean values within and between treatment groups. Number of animals per treatment per time = 5.

3.3.3.3 Angiopoietin-1 protein

Statistical analysis indicated that there was no significant effect of progesterone priming (P = 0.19) nor timing of the LH surge (P = 0.95) on Ang-1 protein expression. However, there were significant effects (P<0.05) of follicular cell layers on Ang-1 protein expression levels as well as significant interaction between factors.

The theca interna expressed significantly higher (P<0.05) overall mean levels of Ang-1 protein than the granulosa and theca externa layers, with no significant difference being observed between the granulosa and theca externa layers (Figure 43).

The significant interaction was due to the fact that while there was no significant difference in Ang-1 protein expression levels between progesterone primed and non-

primed animals in the different layers before the LH surge, after the LH surge progesterone primed animals showed significantly higher ($P<0.05$) Ang-1 protein expression levels in the theca interna layer when compared to non-primed animals (Figure 44).

Mean Ang-1 protein values observed in the different cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 14.

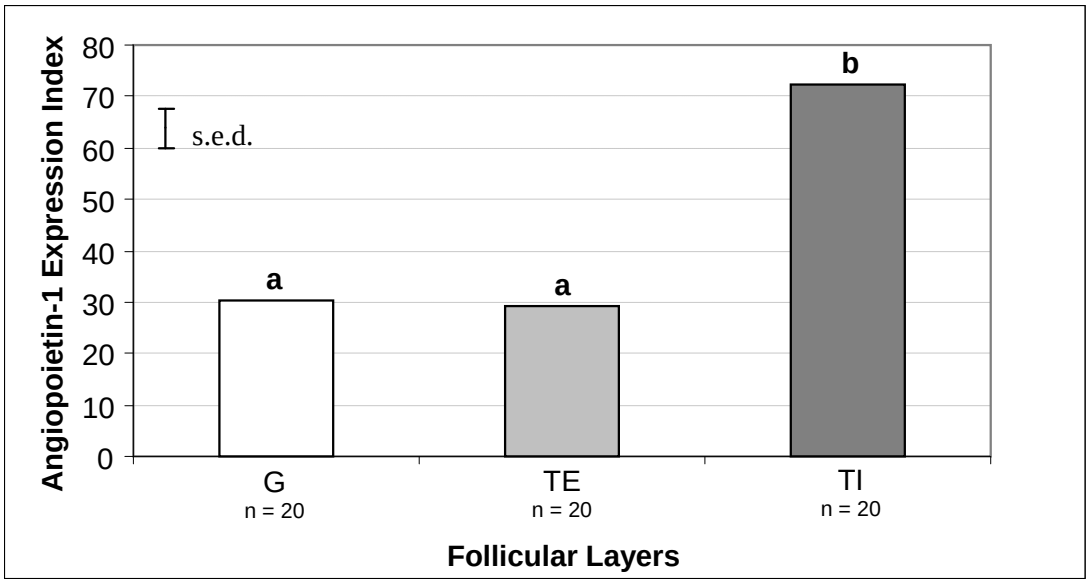

Figure 43. The overall mean Expression Index of Angiopoietin-1 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 3.876, standard error of difference between means to compare the mean values for Angiopoietin-1 expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 14. Mean Angiopoietin-1 protein Expression Index values observed in the various cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	35.93	23.07	63.78
2	+	Pre-LH	33.28	42.31	68.54
3	-	Post-LH	13.90	17.27	58.64
4	+	Post-LH	37.48	34.18	98.97

s.e.d. (18.17) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

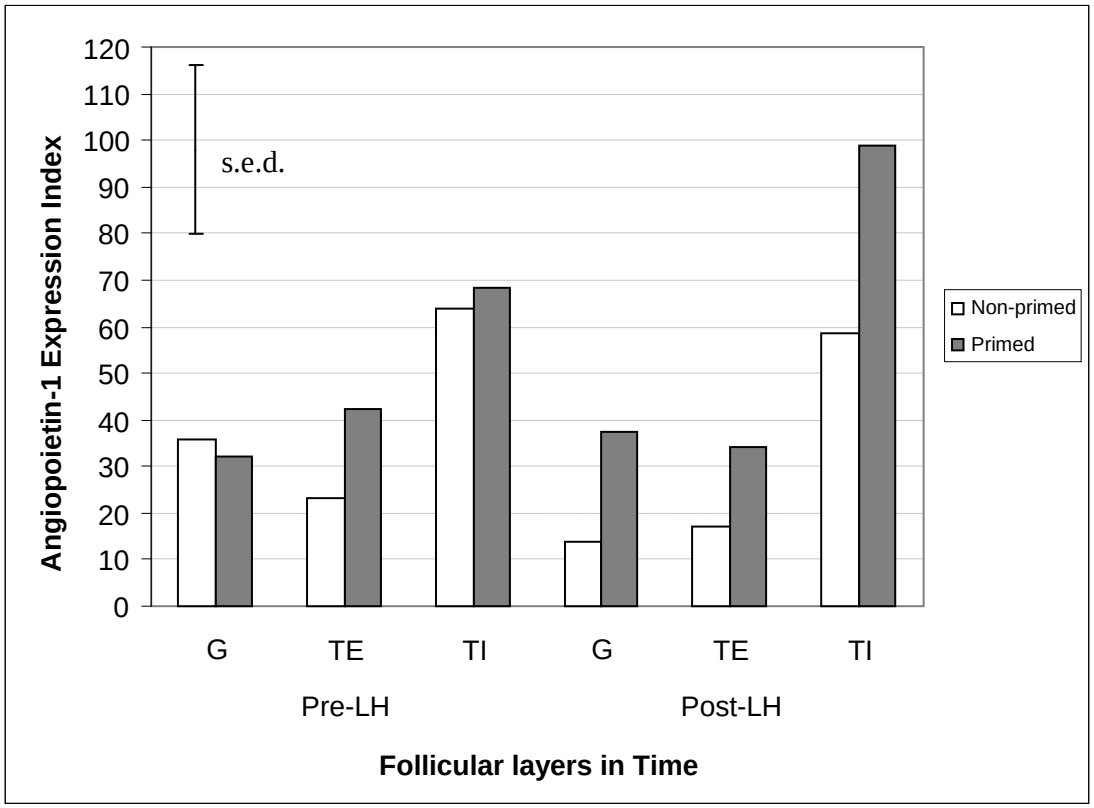

Figure 44 The mean Expression Index of Angiopoietin-1 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, prior and post-LH surge. The s.e.d. = 18.17, standard error of the difference between means to compare mean values within and between treatment groups. Number of animals per treatment per time = 5.

3.3.3.4 Angiopoietin-1 mRNA

As data was not normally distributed, values were submitted to Square Root transformation prior to statistical analysis. There was no significant effect of progesterone priming ($P = 0.17$) nor timing of the LH surge ($P = 0.40$) on Ang-1 mRNA expression levels. However, there were significant effects ($P<0.05$) of follicular cell layers on Ang-1 mRNA expression levels as well as significant interaction between factors.

The granulosa showed significantly higher ($P<0.05$) overall mean levels of Ang-1 mRNA expression than both theca layers, and the theca interna showed significantly higher ($P<0.05$) levels of Ang-1 mRNA expression than the theca externa (Figure 45).

While no significant difference was observed in Ang-1 mRNA levels in the different cell layers of progesterone primed and non-primed animals before the LH surge, progesterone primed animals showed significantly higher ($P<0.05$) mean levels of Ang-1 mRNA than non-primed animals but only in the theca interna layer after the LH surge. It was this factor that was responsible for the significant interaction (Figure 46).

Mean Ang-1 mRNA values observed in the different cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with GnRH are detailed in Table 15.

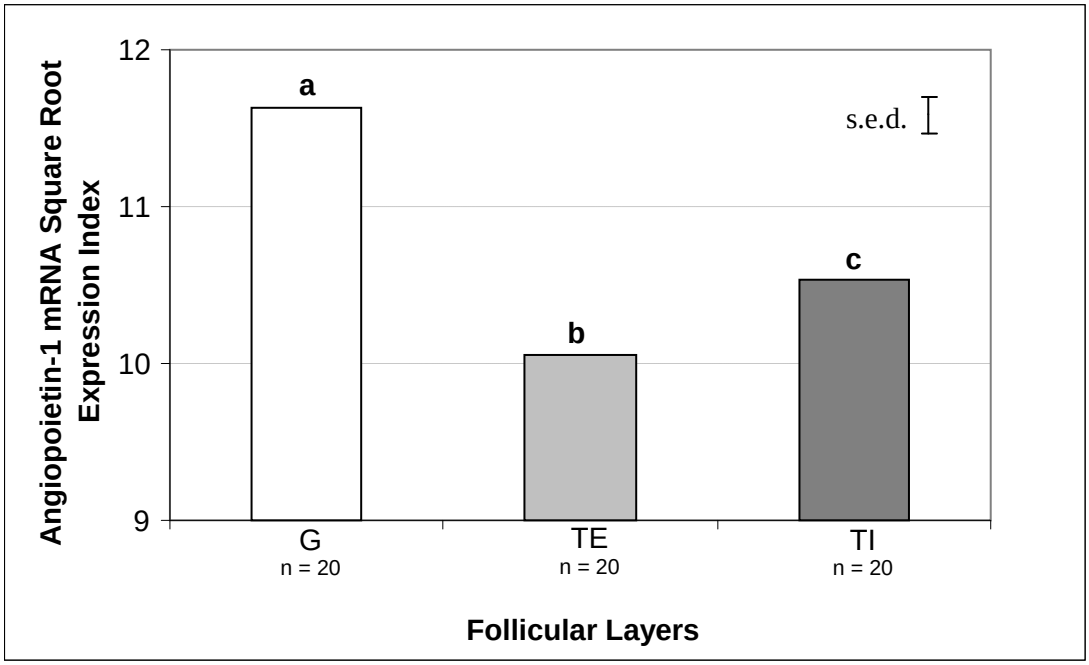

Figure 45. The overall mean Square Root Expression Index of Angiopoietin-1 mRNA in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. Square Root s.e.d. = 0.120, standard error of difference between means to compare the mean values for Angiopoietin-1 mRNA expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 15. Back transformed mean values for Angiopoietin-1 mRNA Expression Index (Square Root values) observed in the various cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	134.79 (11.61)	100.80 (10.04)	104.65 (10.23)
2	+	Pre-LH	134.56 (11.60)	102.21 (10.11)	104.85 (10.24)
3	-	Post-LH	131.10 (11.45)	101.00 (10.05)	105.26 (10.26)
4	+	Post-LH	140.42 (11.85)	100.20 (10.01)	129.27 (11.37)

Square Root s.e.d. (0.297) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

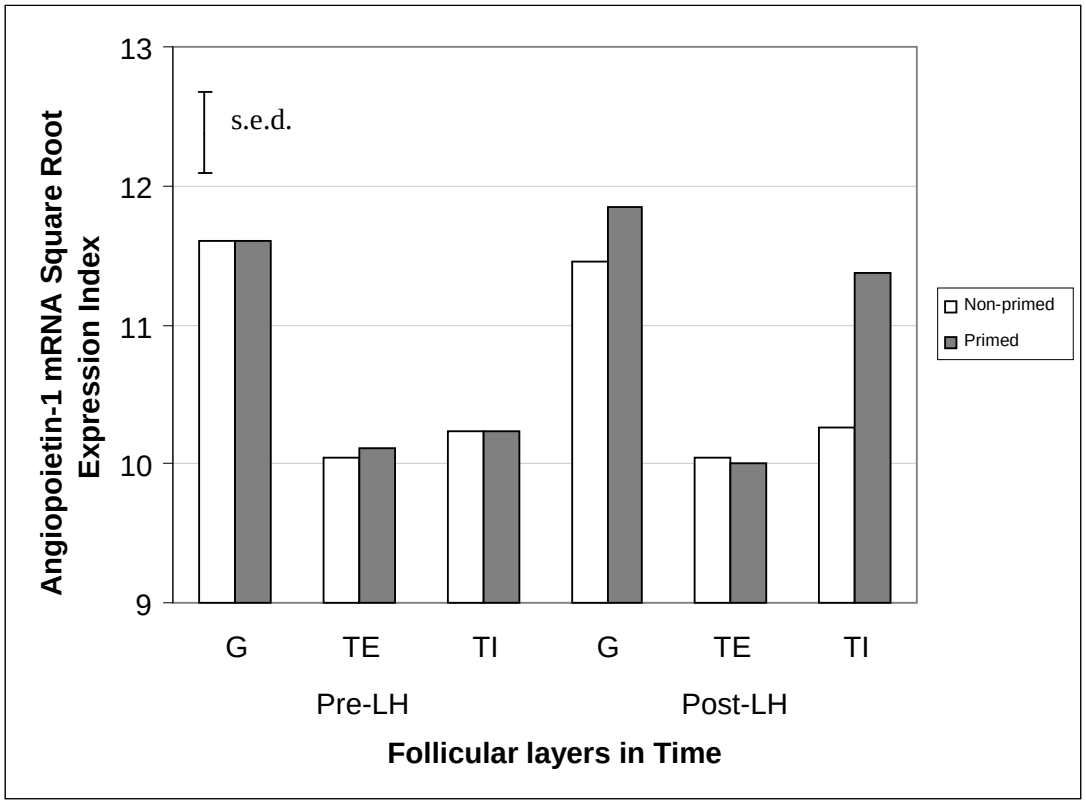

Figure 46. The mean Square Root Expression Index of Angiopoietin-1 mRNA in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, prior and post-LH surge. The s.e.d. = 0.297, standard error of the difference between means to compare mean values within and between treatment groups. Number of animals per treatment per time = 5.

3.3.3.5 Angiopoietin-2 protein

Statistical analysis indicated that there was no significant effect of progesterone priming ($P = 0.42$) nor timing of the LH surge ($P = 0.89$) on Ang-2 protein expression levels. However, there were significant effects ($P<0.05$) of follicular cell layers on Ang-2 protein expression levels as well as significant interaction between factors.

The theca interna expressed significantly higher ($P<0.05$) overall mean levels of Ang-2 protein than the granulosa and theca externa layers, with no significant difference being observed between the granulosa and theca externa layers (Figure 47).

The significant (P<0.05) interaction was attributed to the high mean levels of Ang-2 protein expression in the theca interna of progesterone primed animals after the LH surge, with no significant differences in any other layer before or after the LH surge (Figure 48).

Mean Ang-2 protein values observed in the different cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with GnRH are detailed in Table 16.

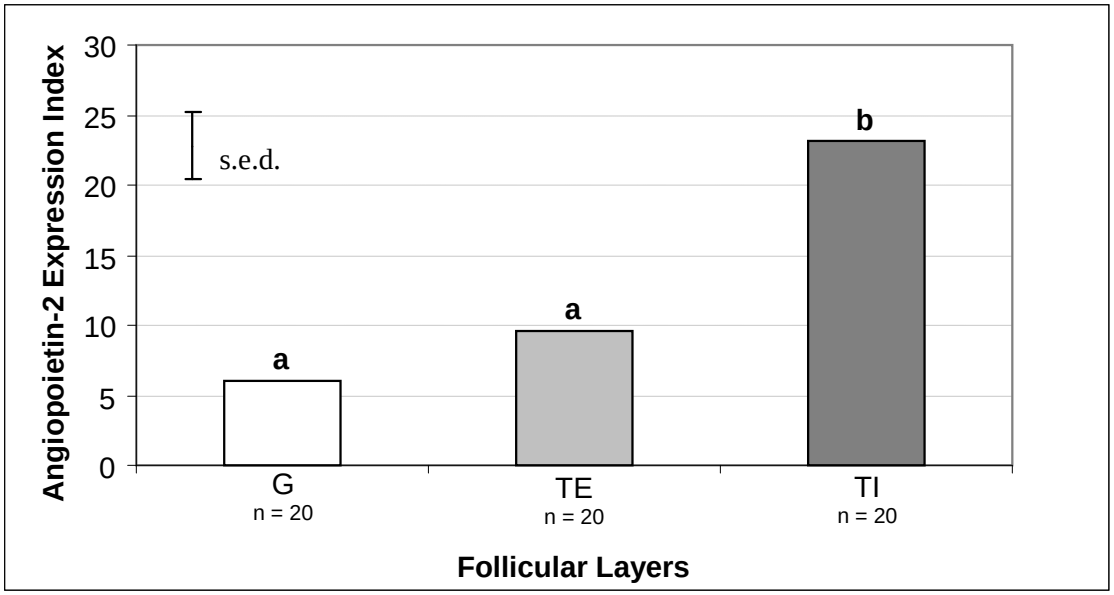

Figure 47. The overall mean Expression Index of Angiopoietin-2 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 2.393, standard error of difference between means to compare the mean values for Angiopoietin-2 expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 16. Mean Angiopoietin-2 protein Expression Index values observed in the various cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	7.53	7.62	26.28
2	+	Pre-LH	10.23	11.73	19.45
3	-	Post-LH	0.93	4.16	13.92
4	+	Post-LH	5.53	14.86	32.95

s.e.d. (8.94) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

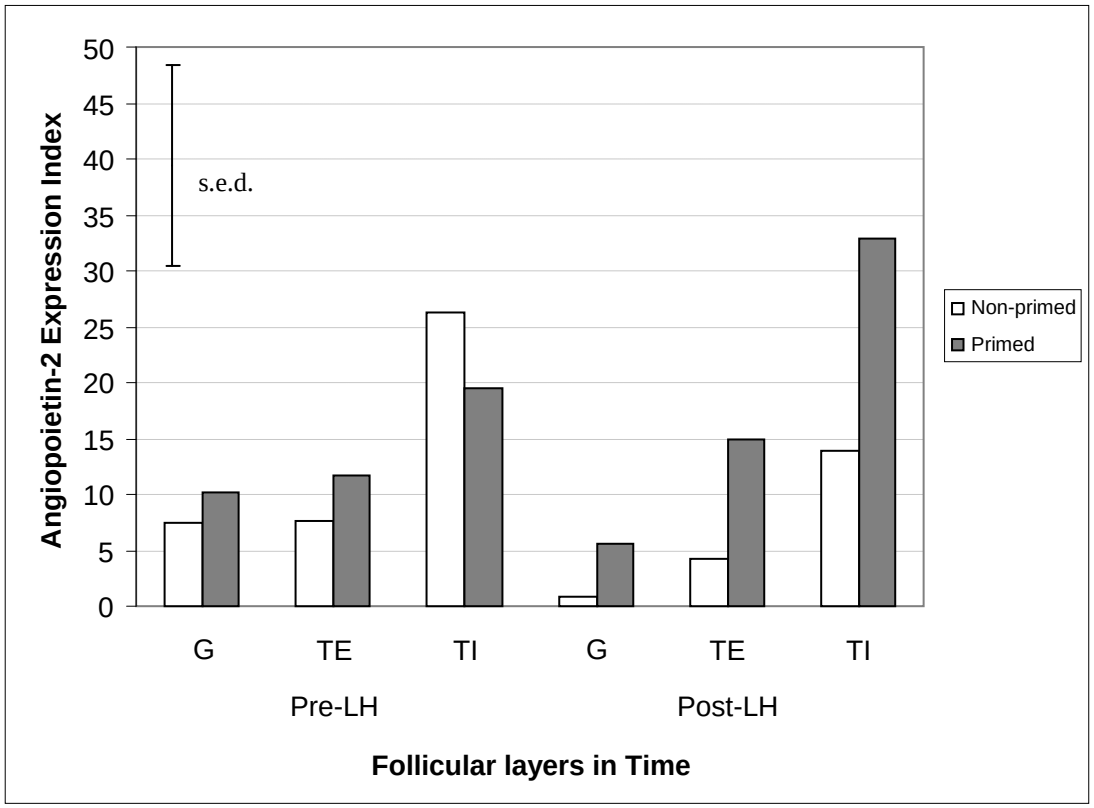

Figure 48. The mean Expression Index of Angiopoietin-2 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, prior and post-LH surge. The s.e.d. = 8.943, standard error of the difference between means to compare mean values within and between treatment groups. Number of animals per treatment per time = 5.

3.3.3.6 Angiopoietin-2 mRNA

Statistical analysis indicated that there was no significant effect of progesterone priming (P = 0.44) nor timing of the LH surge (P = 0.12) on Ang-2 mRNA expression levels. However, there was a significant effect (P<0.05) of follicular cell layers on Ang-2 mRNA expression levels but with no significant interaction between factors (P = 0.06).

The granulosa showed significantly higher (P<0.05) overall levels of Ang-2 mRNA than the theca layers, with no significant difference being observed between the theca interna and theca externa layers (Figure 49).

Mean Ang-2 mRNA values observed in the different cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 17.

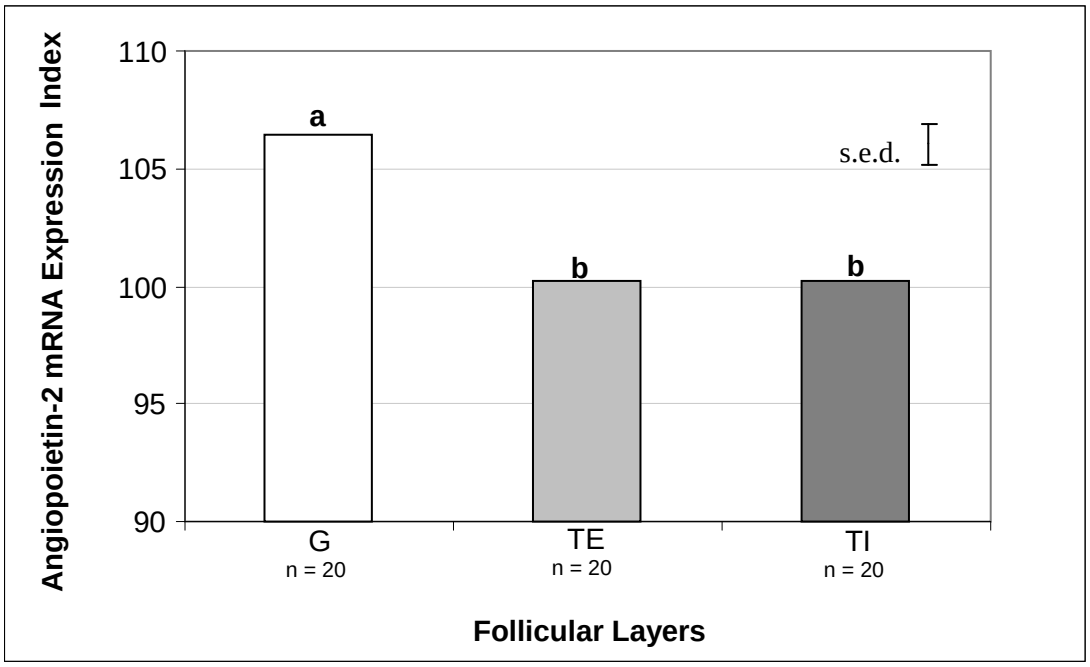

Figure 49. The overall mean Expression Index of Angiopoietin-2 mRNA in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 0.884, standard error of difference between means to compare the mean values for Angiopoietin-2 mRNA expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 17. Mean Angiopoietin-2 mRNA Expression Index values observed in the various cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	111.8	100.5	100.5
2	+	Pre-LH	105.0	100.2	100.2
3	-	Post-LH	104.1	100.0	100.0
4	+	Post-LH	104.9	100.0	100.0

s.e.d. (2.16) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

3.3.3.7 TIE-2 protein

Statistical analysis indicated that there was no significant effect of progesterone priming ($P = 0.33$) nor timing of the LH surge ($P = 0.41$) on TIE-2 protein expression levels. However, there was a significant effect ($P<0.05$) of follicular cell layers on TIE-2 protein levels but with no significant interaction between factors ($P = 0.63$).

The theca externa layer expressed significantly higher ($P<0.05$) overall mean levels of TIE-2 protein than the granulosa, with no significant difference between the theca interna and theca externa layers nor between the granulosa and theca interna layer (Figure 50).

Mean TIE-2 protein expression values observed in the different cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 18.

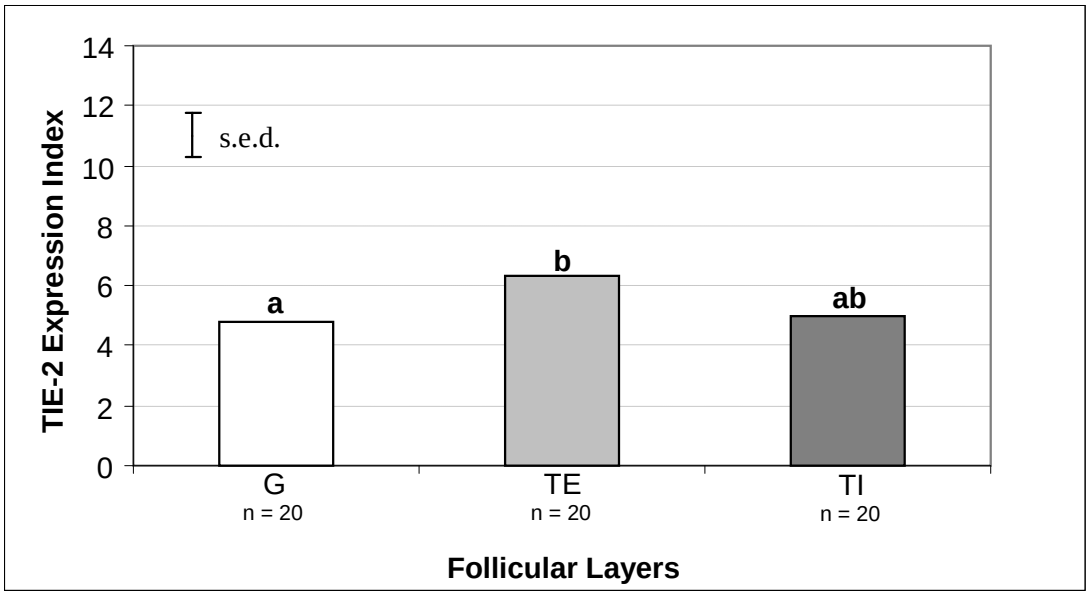

Figure 50. The overall mean Expression Index of TIE-2 protein in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 0.748, standard error of difference between means to compare the mean values for TIE-2 expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 18. Mean TIE-2 protein Expression Index values observed in the various cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	6.438	8.856	8.536
2	+	Pre-LH	4.134	5.316	2.405
3	-	Post-LH	3.811	5.905	4.785
4	+	Post-LH	4.898	5.181	4.181

s.e.d. (2.82) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

3.3.3.8 TIE-2 mRNA

Statistical analysis indicated that there was no significant effect of progesterone priming (P = 0.14) nor timing of the LH surge (P = 0.75) on TIE-2 mRNA expression levels. However, there were significant effects (P<0.05) of follicular cell layers on TIE-2 mRNA expression levels as well as a significant interaction between factors.

The granulosa expressed significantly higher (P<0.05) overall mean levels of TIE-2 mRNA than the theca layers, with no significant difference between the theca interna and theca externa layers (Figure 51).

In terms of the significant interaction, TIE-2 mRNA expression before the LH surge was significantly higher (P<0.05) in the granulosa and theca externa layers of progesterone primed animals compared to non-primed. This difference, however, disappeared by the post-LH period (Figure 52).

Mean TIE-2 mRNA values observed in the different cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH are detailed in Table 19.

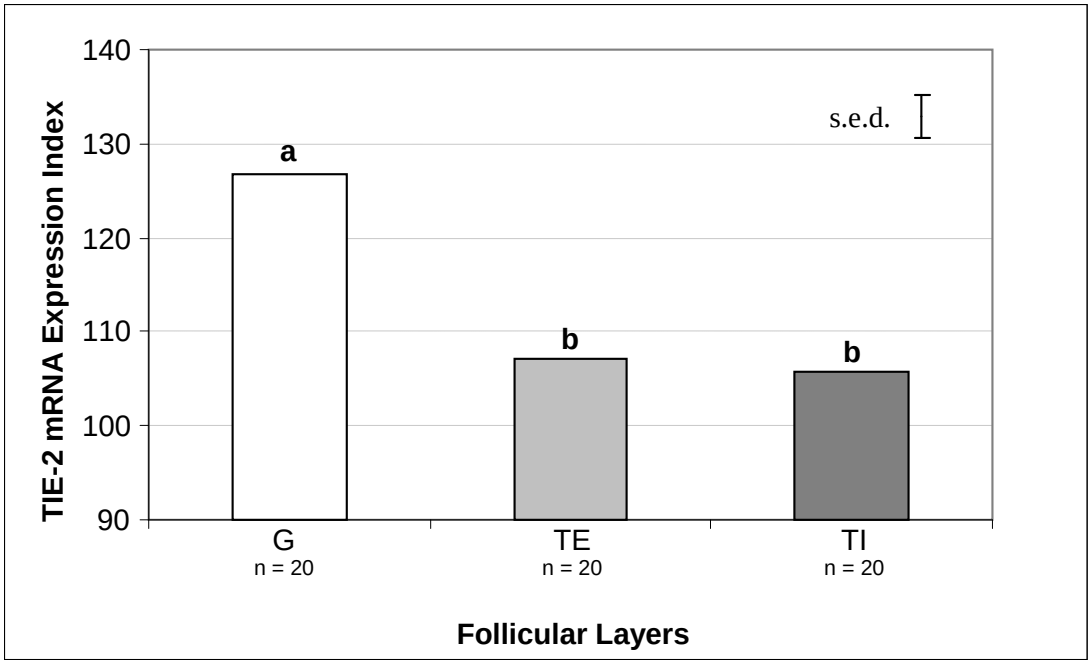

Figure 51. The overall mean Expression Index of TIE-2 mRNA in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles of seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. s.e.d. = 2.237, standard error of difference between means to compare the mean values for TIE-2 mRNA expression between cell layers. Different letters on bars indicate significant difference at 5% level. n = number of animals.

Table 19. Mean TIE-2 mRNA Expression Index values observed in the various cell layers of large follicles before and after the LH surge from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH.

Groups	Progesterone	Time	Layer		
			Granulosa	Theca externa	Theca interna
1	-	Pre-LH	119.6	100.7	100.8
2	+	Pre-LH	138.5	119.4	106.1
3	-	Post-LH	122.8	101.9	103.1
4	+	Post-LH	125.8	106.3	112.9

s.e.d. (8.70) = standard error of difference between means to compare the mean values both within and between the various treatment groups.

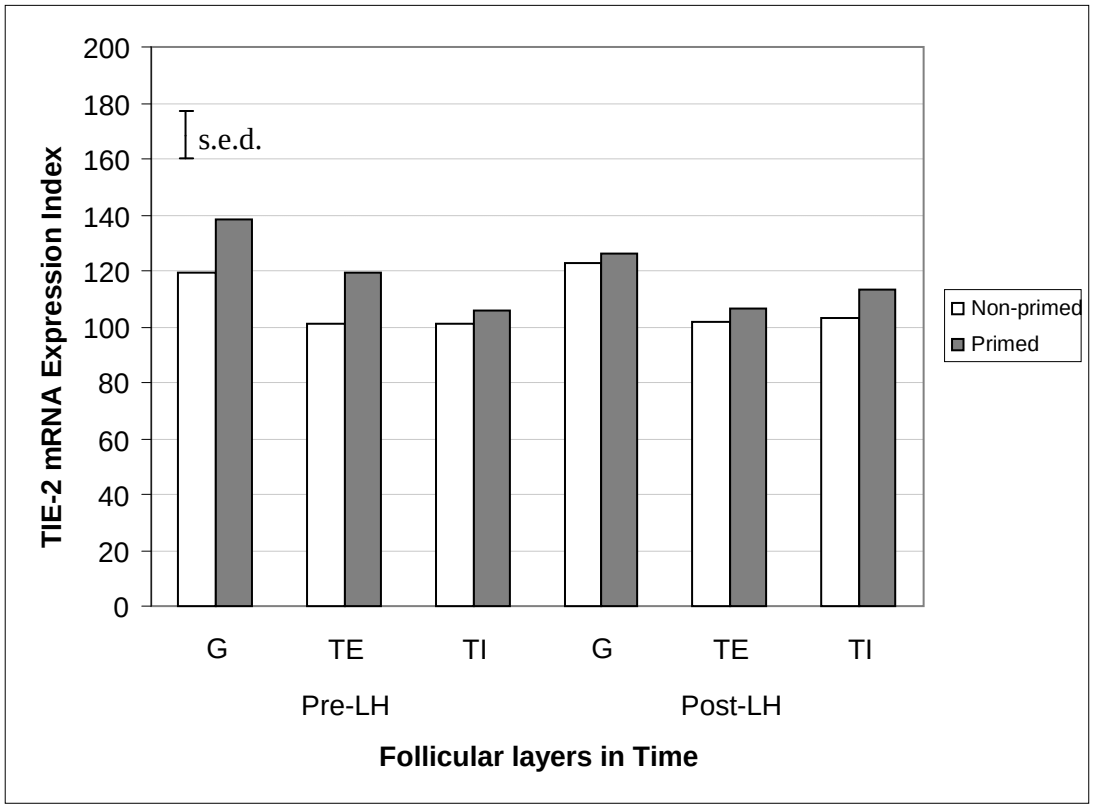

Figure 52. The mean Expression Index of TIE-2 mRNA in the granulosa (G), theca externa (TE) and theca interna (TI) layers of large follicles from progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH, prior and post-LH surge. The s.e.d. = 8.701, standard error of the difference between means to compare mean values within and between treatment groups. Number of animals per treatment per time = 5.

A summary of the effects on angiogenic factors levels before and after the LH surge in the granulosa, theca interna and theca externa layers of both small and large follicles of progesterone primed and non-primed animals treated with small-dose multiple injections of GnRH can be seen in Table 20.

Summary table

3.4 Discussion

It has been reported that seasonally anoestrous ewes pre-treated with progesterone prior to GnRH-induced ovulation show an LH surge significantly later than non-primed animals (39 versus 20 hours after the start of GnRH treatment, respectively) when progesterone priming was removed at the start of GnRH treatment (McLeod et al., 1982b). However, when progesterone injection is given 5 or 3 days before the start of GnRH treatment, as in the case of this study, there is no delay in the onset of the LH surge (Pearce et al., 1987). Furthermore, it has been shown that when given intravenously, a GnRH bolus is able to immediately induce an LH surge (Hunter et al., 1988). Progesterone priming has no effect on the duration or amplitude of the LH surge (Haresign et al., 1996, Legan et al., 1985), therefore, once the pre-ovulatory LH surge is induced there should be no significant variation between primed and non-primed animals in the time required for ovulation to occur. Ovulation following GnRH bolus injection is expected to occur 30 hours after the onset of the LH surge (Stellflug et al., 1997, Bazer et al., 1994), 60 hours after the beginning of GnRH treatment in this study.

In this project a 300 μg GnRH bolus injection given intravenously 30 hours after the start of the GnRH treatment resulted in synchronization of the onset of the LH surge in all animals, as reported previously by Khalid *et al.* (1997). GnRH bolus injection was designed at 30 hours after the start of pulsatile GnRH treatment in order to expose follicles to a sustained increase in LH for a period long enough for their development and maturation. Moreover, the synchronization of the onset of the pre-ovulatory LH surge was of extreme importance in order to establish ovary collection times and guarantee that follicles were exposed to gonadotrophin stimulation for the same length of time in both treatment groups. Based on the LH profile for each group it is safe to say that ovaries from Group 1 and Group 2 animals were collected prior to the LH surge (24 hours after start of GnRH treatment) and ovaries from Group 3 and Group 4 were collected after the LH surge but prior to ovulation (46 hours after the start of GnRH treatment). Therefore, post-LH surge ovaries were collected 16 hours after the onset of the LH surge but 14 hours prior to the expected ovulation time. The lack of ovulation was further confirmed by the

absence of a *corpus haemorrhagicum* and/or an ovulation point in the majority of animals. The ovulated animals (1/10 from primed and 2/10 from non-primed groups) were excluded from any analysis.

Adequate ovarian vasculature is essential for follicular development and ovulation (Shimizu et al., 2007). Furthermore, the acquisition of adequate vascular supply is essential for the delivery of hormones and nutrients to the developing follicle prior to ovulation (Tamanini and De Ambrogi, 2004). The theca blood vessels are responsible for providing the growing follicles with gonadotrophins, growth factors, steroid precursors and other substances essential for adequate vascular development (Kaczmareck et al., 2005). Although blood vessels are limited to the theca layers, the granulosa cells seem to be responsible for a significant production of angiogenic factors (Tamanini and De Ambrogi, 2004) which can diffuse to the theca compartment. These factors are also thought to accumulate in the follicular fluid creating an angiogenic gradient attracting blood vessels closer to the granulosa layer (Mattioli et al., 2001).

VEGF is essential for follicular development (Hunter et al., 2004, Ferrara et al., 2003a) and the persistence of the theca interna capillary network is directly dependent on VEGF accumulation in the follicular fluid (Mattioli et al., 2001). VEGF is also necessary for antrum development and for neovascularisation to occur (Hayashi et al., 2003).

In small follicles there was a significant difference in VEGF protein expression by the different cell layers. Although the granulosa layer is said to be the main source of VEGF production in the follicle (Hunter et al., 2004, Robinson et al., 2007), in this study it was observed that in small follicles of 2-2.5 mm in diameter, the theca interna expressed significantly higher levels of VEGF protein than the theca externa or the granulosa layer. As the theca interna is the main source of vascular supply to the developing follicle (Mattioli et al., 2001), high levels of VEGF are expected to be present in such follicles as follicular angiogenesis is still taking place during early follicular development.

Progesterone priming did not have any significant effect on VEGF expression in small follicles before or after the LH surge.

Although it has been reported that LH stimulates VEGF expression in the granulosa layer (Tamanini and De Ambrogi, 2004), VEGF levels in small follicles were found to be significantly higher prior to the LH surge. LH receptors are known to appear in the granulosa cells of ovine follicles when they reach approximately 3.5 mm in diameter (Hunter et al., 2004). As all small follicles analyzed in this study ranged from 2 to 2.5 mm in diameter, LH receptors may well not have been present in the granulosa layer. This observation leads to the conclusion that the LH surge does not have a direct influence in upregulating VEGF levels in small follicles. Furthermore, after the LH surge, small follicles either have their development halted or undergo atresia (Hunter et al., 2004, Driancourt, 2001, Stabenfeldt and Edquist, 1996). In either case, VEGF levels are expected to decline (Wulff et al., 2001a), as observed in this study.

VEGFR-2 was found to be expressed mainly by the theca layers of small follicles, as previously reported (Wulff et al., 2001a, Hunter et al., 2004). The highest levels of VEGFR-2 were found in the theca interna while the lowest was observed in the granulosa layer. As with VEGF protein, progesterone priming had no overall effect on VEGFR-2 levels, but unlike with VEGF, the LH surge did not significantly alter VEGFR-2 levels in small follicles.

Angiopoietins act in association with VEGF to regulate blood vessel formation and maturation (Davis et al., 1996). In small follicles it was observed that all cell layers were capable of expressing Ang-1 protein and mRNA. The theca interna layer expressed significantly higher levels of Ang-1 protein than the theca externa or granulosa layers. The granulosa showed the lowest amounts of Ang-1 protein of all follicular layers. Ang-1 mRNA, on the other hand, was mainly expressed by the granulosa layer, followed by the theca interna and last by the theca externa layer. These findings are in agreement with previous reports on Ang-1 mRNA expression in the follicular layers (Hayashi et al., 2003, Shimizu et al., 2007). This difference in protein and mRNA expression by the different follicular layers could possibly be explained by the diffusion of the protein from the granulosa to the theca layer (Mattioli et al., 2001) or to the follicular fluid.

Neither progesterone priming nor the LH surge had any significant effect on Ang-1 protein or mRNA levels in small follicles.

Similarly to Ang-1, Ang-2 protein in small follicles was found at its highest in the theca interna and lowest in the granulosa layer. Ang-2 mRNA levels were found relatively evenly in all three follicular layers, with the granulosa layers showing slight but significantly higher amounts of Ang-2 mRNA. These findings are in agreement with previously reported data in which both theca and granulosa layers are capable of expressing Ang-2 mRNA (Hayashi et al., 2003, Shimizu et al., 2007).

Neither progesterone priming nor the LH surge had any overall significant effect on either Ang-2 protein or mRNA expression in small follicles.

TIE-2 expression did not significantly vary between the follicular cell layers of small follicles. Interestingly, TIE-2 mRNA was found to be significantly higher in the granulosa than in the theca layers. This difference in TIE-2 mRNA expression, but not in protein concentration, in the different follicular layers could be explained by the fact that TIE-2 protein produced by the granulosa cells has likely diffused to the theca compartment, accounting for the lack of variation in protein levels between the follicular layers. It has been reported that in bovine follicles TIE-2 mRNA can only be found in the theca layers (Shimizu et al., 2007, Hayashi et al., 2003), however, in other species such as primates TIE-2 mRNA was found to be present in the granulosa as well as the theca layers (Wulff et al., 2001a). We have observed that ovine follicles express TIE-2 mRNA in all 3 follicular layers.

Progesterone priming did not have any significant effect on either TIE-2 protein levels or mRNA expression in small follicles. Furthermore, TIE-2 protein and mRNA did not significantly vary before or after the LH surge.

These findings lead to the conclusion that LH has no influence on either Angiopoietin or their receptor in small follicles and that these relatively low levels associated with low VEGF post-LH surge result in regression of vascularity leading to a halt in follicular development and/or atresia.

Based on these findings it can be concluded that the negative effect that the lack of progesterone priming prior to GnRH treatment has on luteal functionality is not related to lower production or expression of angiogenic factors or their receptors in small follicles before or after the LH surge. This is expected as follicles up to 2.5 mm just before the LH surge are not supposed to ovulate, and this size of follicles

which had already been exposed to an LH surge would certainly undergo atresia and halt any further development.

The great majority of the large follicles used in this project (62%) were ≥3.5 mm and therefore would be expected to express LH receptors in the granulosa layer (Hunter et al., 2004) and become more susceptible to LH than small follicles.

Interestingly, VEGF production by large follicles was highest in the theca interna layer, as observed in small follicles. As the majority of the follicles were expected to have developed LH receptors in the granulosa layer, it was expected that LH would have stimulated enough VEGF production by the granulosa layer to turn it into the main source of VEGF protein in the follicle, as reported previously (Hunter et al., 2004, Robinson et al., 2007). However, the lower VEGF levels observed in the granulosa layer may still accumulate and result in high VEGF levels in the follicular fluid which would act as a stimulus for blood vessels to extend towards the granulosa around the time of ovulation (Mattioli et al., 2001). Furthermore, VEGF levels produced by the granulosa could be diffusing into the theca interna layer and thus accounting for the elevated levels of protein observed in that layer.

Non-primed animals showed significantly lower overall VEGF levels than progesterone primed animals. As the theca capillary network is directly dependent on VEGF levels, the lower VEGF levels observed in non-primed animals may lead to degradation of the existing vascular bed and poor sprouting around the time of ovulation. Low VEGF levels during the late follicular phase have been shown to interfere with dominant follicle ovulation and have been associated with the development of non-functional CL (Fraser and Duncan, 2009).

VEGF levels in large follicles were found to be significantly higher before the LH surge, as observed in small follicles. This is in agreement with previously reported data in which VEGF levels decreased at peri-ovulatory time (Fraser and Duncan, 2009), however, the levels observed after the LH surge may be sufficient to induce angiogenesis. During the peri-ovulatory period VEGF levels are known to increase markedly in the follicular fluid (Ferrara et al., 1992, Tamanini and De Ambrogi, 2004). Even though lower levels of VEGF were observed in the different cell layers, VEGF levels could possibly be found to be high in follicular fluid as expected because of the accumulation from the high levels prior to the LH surge.

However, follicular fluid measurements of angiogenic factors were not part of this study.

VEGFR-2 levels were found to be significantly higher in the theca interna of large follicles when compared to the granulosa and theca externa layers. The LH surge did not have any significant overall influence on VEGFR-2 protein levels in large follicles.

No significant change was observed in VEGFR-2 levels in the granulosa and theca externa layer of both progesterone primed and non-primed animals before or after the LH surge, remaining relatively low. VEGFR-2 expression in the theca interna layer, on the other hand, showed dramatic variation. While progesterone primed animals showed a significant increase in VEGFR-2 levels after the LH surge, non-primed animals showed a significant decrease in receptor levels. Furthermore, VEGFR-2 levels observed in the theca interna of progesterone primed animals after the LH surge were significantly higher than those observed in non-primed animals.

Around the time of the LH surge the basal membrane between the granulosa and theca interna layers dissolves and the theca capillaries expand and sprout into the avascular granulosa layer to form a dense vascular network which will be the base for luteal vascular development (Shimizu et al., 2007). Significantly lower levels of VEGF and VEGFR-2 observed in non-primed animals could significantly interfere with blood vessel sprouting, thereby limiting early luteal angiogenesis.

Progesterone seems to act in synergism with LH to upregulate VEGFR-2 production by the theca interna cells of large follicles. LH is known to stimulate VEGF expression (Tamanini and De Ambrogi, 2004) and this study has shown that it has a similar effect on VEGFR-2 production. Although it is not clear why the LH surge did not result in an increased expression of VEGF in large follicles, an increase in the VEGFR-2 expression by the theca interna cell of progesterone primed animals may result into an overall increased effect of VEGF and therefore, a higher degree of vascularisation in these animals.

In large follicles all 3 layers were able to express Ang-1 protein. However, the theca interna showed significantly higher levels of Ang-1 than the granulosa and theca externa layers. Furthermore, no significant difference was observed in Ang-1 levels between the granulosa and theca externa layers. As observed in small follicles,

the granulosa layer of large follicles showed significantly higher levels of Ang-1 mRNA expression than the theca layers. Ang-1 mRNA was found at its lowest in the theca externa layer.

In both small and large follicles, Ang-1 protein was mainly produced by the theca interna while Ang-1 mRNA was found at its highest levels in the granulosa layer. Although both the granulosa and the theca layers have been reported to express mRNA for Ang-1 (Shimizu et al., 2007, Hayashi et al., 2003), it is nevertheless interesting to observe that protein production did not always reflect mRNA expression, whether in concentration or location.

Neither progesterone priming nor the LH surge had any significant overall effect on Ang-1 protein or mRNA expression. Ang-1 mRNA has been shown to be highly expressed in bovine follicles before the LH surge followed by a significant decrease at the time of the LH surge and a slow and consistent rise after the LH surge (Shimizu et al., 2007). Although there is fluctuation in Ang-1 mRNA expression before and after the LH surge, the lack of variation in Ang-1 mRNA levels observed in this study could be due to the collecting times coinciding with the high levels of Ang-1 mRNA expression by the follicle, both before and after the LH surge.

No significant alterations in overall Ang-1 protein levels were observed in any of the cell layers before or after the LH surge, however, Ang-1 levels were significantly higher in the theca interna of progesterone primed animals after the LH surge than those observed in non-primed animals. Interestingly, Ang-1 mRNA levels did not vary in any layer of progesterone primed and non-primed animals before or after the LH surge. Therefore, although Ang-1 mRNA expression in the theca interna of both treatment groups after the LH surge was very similar, they yielded different responses in protein production. While non-primed animals showed similar Ang-1 levels to the pre-LH phase, progesterone primed animals showed significantly higher levels than non-primed ones.

While in small follicles progesterone priming and the LH surge do not seem to affect Ang-1 protein and mRNA expression levels, in large follicles progesterone priming seems to upregulate Ang-1 protein expression by the theca interna cells after the LH surge, possibly contributing to the early luteinisation process that takes place in the cells of the ovulatory follicle (Hayashi et al., 2003).

As with Ang-1, all follicular layers of large follicles were capable of expressing Ang-2 protein. However, the highest Ang-2 levels were observed in the theca interna with no difference observed between the theca externa and granulosa layer. As in small follicles, Ang-2 mRNA was mainly expressed by the granulosa layer. The expression of Ang-2 mRNA by all three follicular layers are in agreement with previously reported data (Hayashi et al., 2003, Tamanini and De Ambrogi, 2004).

Neither progesterone nor the LH surge had any overall significant effect on Ang-2 protein and mRNA expression in large follicles. However, Ang-2 protein levels in the theca interna of progesterone primed animals after the LH surge were significantly higher than those found in non-primed animals. Interestingly, Ang-2 mRNA in the theca interna after the LH surge did not significantly vary between the treatment groups, suggesting that progesterone acts in synergism with LH to upregulate Ang-2 protein production. These findings are in agreement with previous reports in which Ang-2 mRNA expression levels were shown to be steady throughout follicular development and ovulation (Shimizu et al., 2007).

In large follicles TIE-2 protein expression was found mainly in the theca externa layer. As observed in small follicles, TIE-2 mRNA expression was found to be higher in the granulosa layer of large follicles. Although both theca layers expressed TIE-2 mRNA no significant difference was observed between them.

Neither progesterone priming nor LH seems to significantly interfere with TIE-2 protein levels in large follicles. Eventhough TIE-2 mRNA levels prior to the LH surge were significantly higher in the theca externa and granulosa layers of progesterone primed animals no overall significant difference between treatments could be observed. TIE-2 mRNA levels remained relatively similar after the LH surge, suggesting that LH has no significant influence on TIE-2 mRNA expression in the different follicular layers of large follicles.

Tissue growth and development depend upon the establishment of an adequate vascular supply. After the LH surge, the theca and granulosa cells of the pre-ovulatory follicle undergo dramatic changes to form the CL. These rapid changes must be equally accompanied by the rapid development of new blood vessels (Berisha and Schams, 2005, Kaczmareck et al., 2005, Miyamoto et al., 2005, Reynolds et al., 2000, Redmer et al., 2001).

After the LH surge theca capillaries invade and sprout into the avascular granulosa, forming the initial luteal vascular network (Shimizu et al., 2007). Most of the blood vessels of the early CL derive from the theca layer (Zheng et al., 1993) and further vascular development originates from sprouting from this existent vascular network (Kaczmareck et al., 2005, Ferrara et al., 1992, Berisha and Schams, 2005). Therefore, the formation of this initial vascular network is essential for the development of an adequate vascular network that will be able to support the demands of the developing CL.

High VEGF and high Ang-2 levels are essential for neovascularisation to occur (Hayashi et al., 2003). The significantly lower levels of VEGF observed in the large follicles of non-primed animals, associated with significantly lower levels of Ang-2 protein after the LH surge, is potentially detrimental to angiogenesis and new blood vessel formation. This is further aggravated by the significantly lower levels of VEGFR-2 after the LH surge. This could possibly result in poor invasion and sprouting of theca capillaries into the granulosa and consequently result in a restricted and limited luteal vascular network.

Inadequate vascular supply has been suggested as a possible cause of defective luteal function (Southee et al., 1988a). A small vascular network would result in less angiogenesis taking place and therefore in a deficient vascular supply to the developing CL. Furthermore, after the LH surge, Ang-1 and Ang-2 are thought to stimulate early luteinisation and progesterone release from the peri-ovulatory follicle (Hayashi et al., 2003). Significantly lower levels of Ang-1 observed in non-primed animals after the LH surge could further interfere or delay the initial luteinisation process taking place in the follicular layers.

Therefore, the abnormal production of angiogenic factors in large follicles of non-primed animals could lead to inadequate development of the microvasculature of the pre-ovulatory follicle and delayed luteinisation of the follicular layers resulting in defective luteal function.

The effect of progesterone priming in overcoming luteal dysfunction cannot be attributed to altered angiogenic factor levels in small follicles. However, the lack of progesterone priming seems to lead to a defective production of these angiogenic factors in large follicles after the LH surge, potentially compromising luteal vascular development and therefore luteal function.

CHAPTER 4

Protein and mRNA expression of angiogenic factors during early luteal development

4.1 Introduction

The CL is a secretory tissue which originates from the luteinisation of the theca and granulosa cell layers of the pre-ovulatory follicle (Berisha and Schams, 2005, Kaczmareck et al., 2005). Small and large luteal cells together with fibroblasts and vascular cells form the basic structure of the CL (Berisha and Schams, 2005, Sawyer, 1995).

Ovulation induced in seasonally anoestrous ewes with small-dose multiple injections of GnRH result in the formation of a defective CL (Southee et al., 1988a, Southee et al., 1988b, McLeod et al., 1982b). However, with progesterone priming prior to the induction of ovulation, the majority of these CL show normal luteal function (McLeod et al., 1982b, Bartlewski et al., 2001, Hunter et al., 1988, Husein and Kriddli, 2003, Haresign et al., 1996, Southee et al., 1988a). The defective CL formed following pulsatile GnRH treatment is morphologically similar to functionally normal CL, with differences becoming apparent only 5 days after ovulation (Southee et al., 1988b, Hunter et al., 1988). It has been reported that defective CL have lower progesterone content (Hunter et al., 1989) and are paler than functional CLs (Hunter et al., 1988, Southee et al., 1988b), suggesting significantly reduced blood support.

Adequate vascularisation is essential for satisfactory tissue growth and development. For the CL to have normal function, its rapid growth must be accompanied by rapid development of a vascular bed (Redmer et al., 2001, Kaczmareck et al., 2005, Berisha and Schams, 2005).

The early CL is characterized by invasion of capillaries and rapid proliferation of endothelial cells (Zheng et al., 1993). The high proliferation of endothelial cells is responsible for the increased vascularity of the mature CL (Schams and Berisha, 2004, Al-zi'abi et al., 2003). VEGF and VEGFR-2 protein levels are high during early luteal development (Kaczmareck et al., 2005, Tamanini and De Ambrogi, 2004). Ang-2 levels are also high while Ang-1 levels are low, resulting in a high Ang-2:Ang-1 ratio during the initial phases of CL development (Schams and Berisha, 2004, Sugino et al., 2005). High VEGF levels and high Ang-2:Ang-1 ratio result in neovascularisation of the newly formed CL (Hayashi et al., 2003).

On the basis of available literature, a summary demonstrating the expected levels of ovarian angiogenic factors and their effect on luteal angiogenesis during the early and mid-luteal phases and early regression of functional CL can be found in Table 21.

Table 21. Expected levels of VEGF, VEGFR-2, Angiopoietin-1 and -2 and TIE-2 protein and their effect on luteal angiogenesis during normal early and mid-luteal development and early luteal regression.

Factors/Luteal phase	**Early**	**Mid**	**Early regression**
VEGF	high	moderate	low
VEGFR-2	high	moderate	low
Ang-1	low	high	low
Ang-2	moderate	moderate	high
Ang-2:Ang-1 ratio	high	low	high
TIE-2	moderate	moderate/high	moderate
Angiogenesis	neovascularisation	stabilization	regression

The development of a vascular bed incapable of supporting the growing CL has been attributed as one of the causes of defective luteal function (Reynolds and Redmer, 1998, Stellflug et al., 1997). It is thus possible that progesterone priming prior to the induction of ovulation with GnRH is directly or indirectly involved in establishing adequate luteal vascularity through regulation of angiogenic factors and their receptors.

As angiogenesis is at its highest soon after ovulation (Al-zi'abi et al., 2003) and inadequate development of the luteal vasculature is believed to be one of the causes of defective luteal function (Reynolds and Redmer, 1998), the CL from the majority of non-primed animals are expected to show compromised angiogenesis during the early luteal phase. Therefore, VEGF and VEGFR-2 protein levels in these animals are expected to be reduced on day 1 and 2 after ovulation while Ang-2:Ang-1 ratio levels are expected to be moderate when compared to those in CL from progesterone primed animals. By day 4 after ovulation, the majority of these CL are expected to be showing signs of early vascular regression with low levels of VEGF and VEGFR-2 accompanied by moderate to high levels of Ang-2:Ang-1 ratio.

An abnormal production of ovarian angiogenic factors during early CL development could lead to an insufficient blood vessel network being established in the newly formed CL. This inadequate blood supply would limit CL growth and

development and most likely influence luteal functionality. Furthermore, insufficient blood supply would result in reduced delivery of nutrients and progesterone precursors to the developing CL limiting its development and, consequently, progesterone production. Lower progesterone levels would lead to impaired inhibition of oxytocin receptors resulting in premature release of endometrial PGF-2α resulting in defective luteal function and premature regression.

The lack of progesterone priming prior to GnRH-induced ovulation in seasonally anoestrous ewes may result in the abnormal production of angiogenic factors in the early CL leading to inadequate vascular support and consequent defective luteal functionality. The current study was designed to investigate this possibility.

4.2 Materials and Methods

In order to study if the lack of progesterone influences ovarian angiogenic factor production, seasonally anoestrous ewes were primed or non-primed with progesterone prior to pulsatile GnRH treatment using the experimental model described in Chapter 2. Luteal samples were collected at 50 (Groups 5 and 6), 74 (Groups 7 and 8) and 122 hours (Groups 9 and 10) after GnRH bolus injection, which successfully induced an immediate LH peak (see Chapter 3). Ovulation following GnRH bolus injection is expected to occur 30 hours after the onset of the LH surge (Stellflug et al., 1997, Bazer et al., 1994), therefore, samples were collected 1, 2 and 4 days after ovulation so that angiogenic factors levels could be analysed in early CLs, before morphological differences between defective and functional CL became apparent.

Protein expression for VEGF, Ang-1 and -2 and their receptors (VEGFR-2 and TIE-2) were determined by Western Blotting while their gene expression (mRNA detection) was measured by *in situ* hybridization. Luteal vascular density was analysed by immunohistochemistry using antibody against Von Willebrand Factor (VWF), a protein commonly used to establish the degree of vascularity in a tissue.

4.2.1 Western Blotting

Western Blotting assays were performed as described in Chapter 2; however, protein concentration, blotting time and antibody probing were optimized for each protein. For optimization gels were loaded with 25, 50, 75 and 100 μg of protein/well and probed with 1:200, 1:500 and 1:1,000 primary antibody and 1:5,000 and 1:10,000 secondary antibody solutions. Based on the appearance and quality of the bands, optimal quantity of protein, primary and secondary antibody concentrations were selected.

For VEGF assays, 100 μg of total protein/sample was loaded and separated using a 14% resolving gel. Blotting was performed for 90 minutes and membranes blocked in 10% w/v milk for 2 hours. Primary antibody probing took place overnight with 1:350 Rabbit anti-human VEGF (VEGF A20, Santa Cruz Biotechnologies). Secondary antibody probing was done using 1:5,000 Goat anti-rabbit IgG (Perbiosciences) for 1 hour. Films were exposed for 5 minutes.

For VEGFR-2 assays, 75 μg of total protein/sample was used and separated by an 8% resolving gel. Blotting was performed for 105 minutes and blocking in 10% w/v milk took place overnight. Primary antibody probing with 1:500 Rabbit anti-mouse Flk-1 (Flk-1, Santa Cruz Biotechnologies) was performed for 12 hours followed by 1 hour probing with 1:10,000 Goat anti-rabbit IgG (Perbiosciences). Films were exposed for 5 minutes.

Ang-1 assays were performed using 30 μg of total protein/sample and a 10% resolving gel. Blotting took place for 105 minutes and blocking in 10% w/v milk for 2 hours. Membranes were primed with 7 μg/ml Rabbit anti-mouse Ang-1 IgG (Ang11-A, Alpha Diagnostics International) overnight and incubated with 1:5,000 Goat anti-rabbit IgG (Perbiosciences) for 1 hour. Films were exposed for 5 minutes.

Ang-2 assays were performed using 50 μg of total protein/sample and a 10% resolving gel. Blotting was performed for 100 minutes and 10% w/v milk blocking for 2 hours. Primary antibody probing with 1:500 Rabbit anti-human Ang-2 (H-70, Santa Cruz Biotechnologies) was performed overnight and probing with 1:10,000 Goat anti-rabbit IgG (Perbiosciences) was done for 1 hour. Films were exposed for 5 minutes.

For TIE-2 assays 50 μg of total protein/sample was run in a 10% resolving gel. Blotting took place for 90 minutes followed by 2 hours incubation with 10% w/v milk blocker. Membranes were placed in 1:500 Rabbit anti-mouse TIE-2 (C-20, Santa Cruz Biotechnologies) overnight and probed with 1:10,000 Goat anti-rabbit IgG (Perbiosciences) for 1 hour. Films were exposed for 15 minutes.

To each gel, samples from different treatment groups were loaded in equal numbers. Gel to gel variation was eliminated by the same positive control being loaded into each gel. For each angiogenic factor, expression values (band intensities) were calculated in relation to controls.

4.2.1.1 Blocking peptide

To ascertain that the band observed after the assay was indeed VEGF, prior to membrane probing primary antibody against human VEGF (VEGF A20, Santa Cruz Biotechnologies) was incubated with an excess of blocking peptide VEGF (hBA-165, Santa Cruz Biotechnologies) at 1:20 ratio for 2 hours and 30 minutes at 4 °C. The blocking peptide consisted of biologically active pure VEGF protein produced in *E. coli*.

The blocking peptide attaches to the binding sites of the primary antibody, therefore, when the primary antibody is placed with the membrane there should not be enough free antibody to bind to the protein of interest and thus no signal should be detected. If the primary antibody used was not specific to VEGF, it would not bind to the blocking peptide and would still be able to bind to other proteins on the membrane. If a signal was detected then it would be most likely that the protein detected was other than VEGF.

4.2.1.2 Heparinase treatment

In order to determine if the high molecular weight observed for VEGF was due to its affinity to heparin-containing proteoglycans, some samples were submitted to heparinase treatment prior to loading on gel. Samples were incubated with Heparinase I (Sigma-Aldrich) at a concentration of 10U/ml, overnight, at 37 °C.

4.2.2 Immunohistochemistry for Von Willebrand Factor

Immunohistochemistry basic protocol was performed as described in Chapter 2. Primary antibody incubation was done using 1:100 Rabbit anti-human Von Willebrand Factor IgG (Sigma-Aldrich) overnight at 4 °C. Secondary antibody was used at a concentration of 1:25 followed by 1 minute colour development using DAB. For every animal one negative and three positive sections were used, making a total of 27 negative and 81 positive sections being analysed.

4.2.3 *In situ* hybridization

The *in situ* hybridization technique was performed as described in Chapter 2. For every assay, 81 positive (AS) and 27 negative (S) sections were used for each mRNA of interest. Enough riboprobe was made to ensure that each assay could at least be repeated once if needed. Hybridization and colour development times were optimized for each factor and are detailed below.

For Ang-1 assays, slides were incubated with hybridization buffer (HB) for 20 hours. Colour was allowed to develop for 20 hours prior to mounting. For Ang-2 assays, slides were incubated with HB for 21 hours and left to develop colour for 19 hours. TIE-2 hybridization was performed for 19 hours and colour development for a further 19 hours prior to mounting.

VEGFR-2 probes were very limited but enough was made so that CL slides could be done. Slides were left in contact with HB for 22 hours prior to 19 hours of colour development.

4.2.4 Statistical Analysis

Data were analysed by two-way analysis of variance (ANOVA) using progesterone priming and days after ovulation as factors. The least significant difference (LSD) was used as a Post Hoc test for multiple comparisons between means. The overall effects of treatment (progesterone priming) and time (1, 2 and 4 days after ovulation) as well as 2-way interactions between factors (treatment x time)

were analyzed. All analyses were done using GenStat 9th edition (Version 9.1.0.147, Lawes Agricultural Trust, VSN International Ltd). Significance was considered at 5% level.

4.3 Results

The proportion of seasonally anoestrous ewes that ovulated in response to pulsatile GnRH treatment associated with GnRH bolus injection ranged from 14/15 in progesterone primed to 12/15 in non-primed animals.

The mean values observed for the different angiogenic factors in 1-, 2- and 4-day old CL of progesterone primed and non-primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH can be seen in Table 22.

4.3.1 VEGF protein

Initial VEGF assays showed a band around 50 kDa (Figure 51) against the expected 22 kDa under reducing conditions. As variation of gel percentage (from 10 to 16%) did not lead to a more accurate molecular weight estimative, blocking peptide assays were performed.

The membrane exposed to primary antibody/blocking peptide showed no bands (Figure 52) confirming the specificity of the antibody used and that, therefore, the band observed was indeed VEGF, albeit at a different molecular weight than expected.

Samples treated with heparinase still showed a main band around 50 kDa, however, a band around 22 kDa was much more evident in these samples than those not submitted to the treatment (Figure 53).

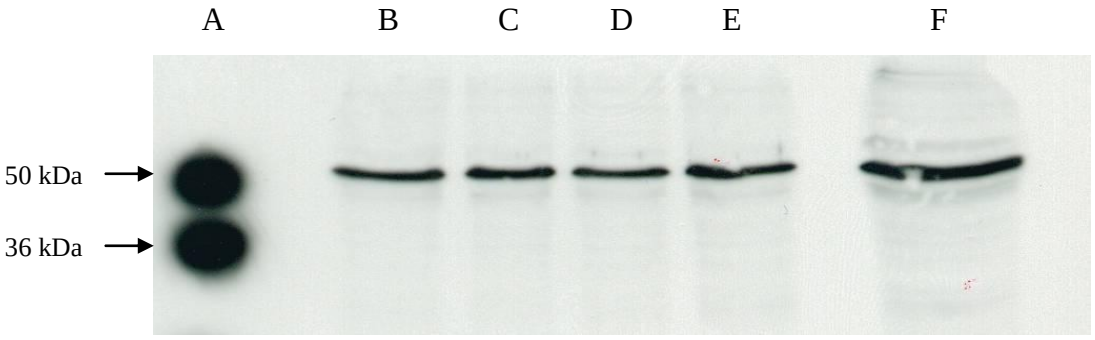

Figure 51. Representative autoradiograph of 10% acrylamide SDS/PAGE VEGF protein immunoblot showing luteal VEGF bands in early corpora lutea from progesterone primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. Bands show control sample (lane F) and luteal samples of 1- (lane D), 2- (lane B and C) and 4- (lane E) day old CL. Molecular weight markers (lane A) of 50 and 36 kDa are indicated by arrows.

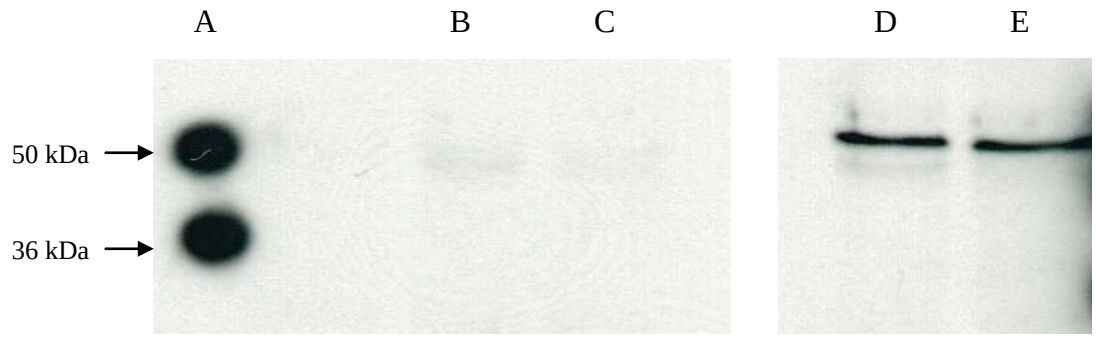

Figure 52. Representative autoradiograph of VEGF protein immunoblot of 4-day old corpora lutea from progesterone primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH, following incubation with (lanes B and C) and without (lanes D and E) blocking peptide. Molecular weight markers (lane A) of 50 and 36 kDa are indicated by arrows.

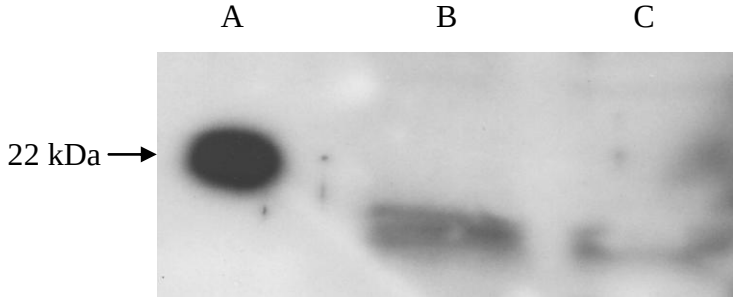

Figure 53. Representative autoradiograph of VEGF protein immunoblot of 4-day old *corpus luteum* from a progesterone primed seasonally anoestrous ewes before (lane C) and after (lane B) Heparinase treatment. Molecular weight marker (lane A) of 22 kDa indicated by arrow.

Although the reducing conditions used in this study did not have the expected effect on luteal VEGF samples, the specificity of the band observed was confirmed by the blocking peptide assay.

No significant difference (P = 0.098) was observed in overall VEGF protein levels between progesterone primed and non-primed animals. A significant difference (P<0.05), however, was observed in VEGF protein levels between the different days after ovulation and the interaction between days and treatment was also significant (P<0.05).

No significant difference was observed between treatment groups either at day 1 or day 4 after ovulation. Both treatment groups showed significantly lower (P<0.05) levels of VEGF at day 1 when compared to days 2 and 4 after ovulation. Non-primed animals displayed similar VEGF levels on day 2 as on day 4 whilst progesterone primed animals showed significantly higher (P<0.05) levels of VEGF at day 2 when compared to day 4 after ovulation (Figure 54).

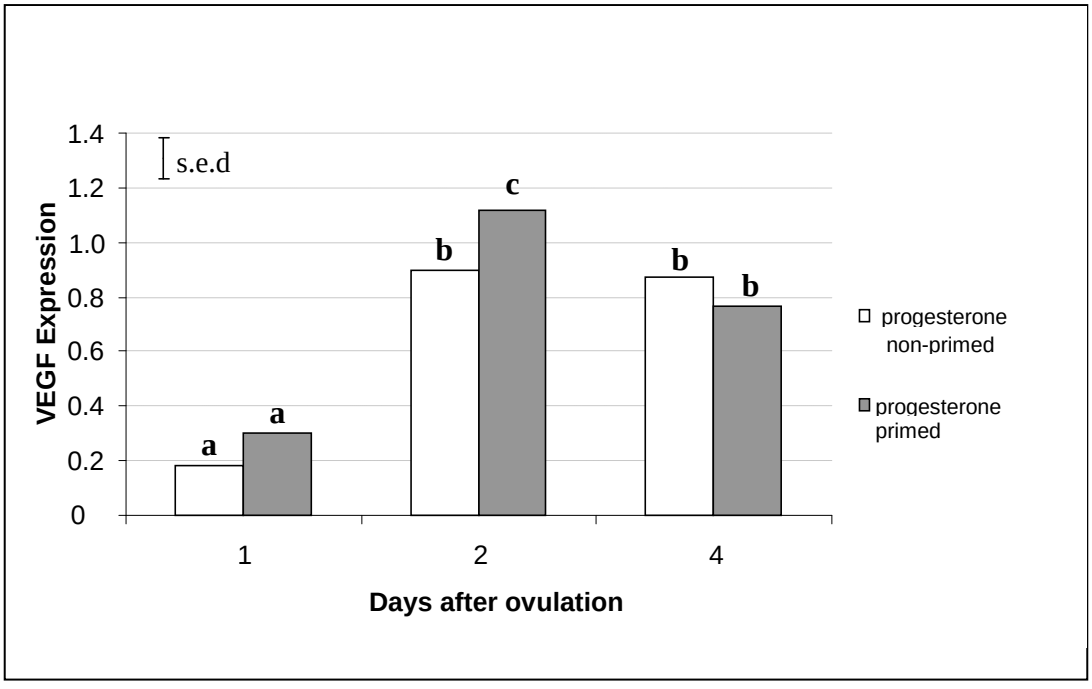

Figure 54. Mean expression of VEGF protein observed 1, 2 and 4 days after ovulation in corpora lutea of progesterone primed and non-primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 0.076, standard error of difference to compare mean values both within and between treatment groups. Different letters on bars indicate significant difference at 5% level. Number of animals per group per day = 5.

4.3.2 VEGFR-2 protein

A significant difference ($P<0.05$) was observed in VEGFR-2 protein levels between progesterone primed and non-primed animals. No significant difference ($P = 0.288$) was observed in VEGFR-2 protein levels between the different days after ovulation, however, a significant ($P<0.05$) interaction was observed between days and treatment.

No significant difference could be observed in luteal VEGFR-2 protein expression between progesterone primed and non-primed animals at day 1 after ovulation. However, progesterone primed animals showed significantly higher ($P<0.05$) levels of VEGFR-2 both at days 2 and 4 when compared to non-primed animals.

Progesterone primed animals maintained a high level of VEGFR-2 which did not vary over time. Non-primed animals, on the other hand, displayed a significant ($P<0.05$) decrease in luteal VEGF levels from day 1 to day 2 and this decline was maintained up to day 4 after ovulation (Figure 55).

VEGFR-2 protein bands were observed just above the 148 kDa molecular weight marker, as expected (Figure 56).

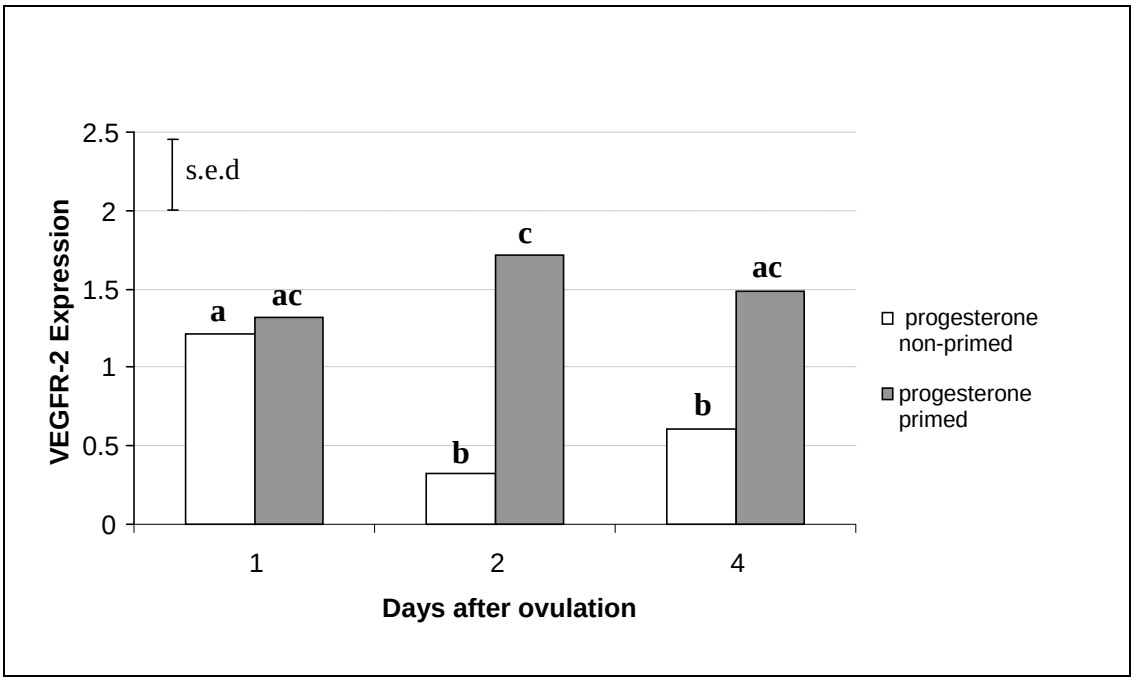

Figure 55. Mean expression of VEGFR-2 protein observed 1, 2 and 4 days after ovulation in corpora lutea of progesterone primed and non-primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 0.225, standard error of difference to compare mean values both within and between treatment groups. Different letters on bars indicate significant difference at 5% level. Number of animals per group per day = 5.

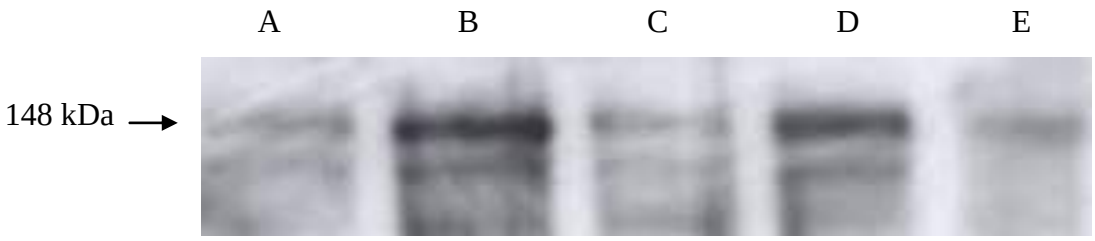

Figure 56. Representative autoradiograph of 8% acrylamide SDS/PAGE VEGFR-2 protein immunoblot showing luteal VEGFR-2 bands of early corpora lutea from progesterone primed and non-primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. Bands illustrate control sample (lane E) and progesterone primed (lane B and D) and non-primed (lanes A and C) luteal samples 2 days after ovulation. Molecular weight marker position is indicated by arrow but not shown.

4.3.3 VEGFR-2 mRNA levels

No significant difference (P = 0.805) was observed in VEGFR-2 mRNA expression levels between progesterone primed and non-primed animals. A significant difference (P<0.05), however, was observed in VEGFR-2 mRNA expression levels between the different days after ovulation with no significant interaction (P = 0.528).

VEGFR-2 mRNA expression levels were significantly higher (P<0.05) on day 1 than on days 2 and 4 after ovulation. No significant difference in VEGFR-2 mRNA expression levels was observed between days 2 and 4 after ovulation (Figure 57).

VEGFR-2 sense and anti-sense images of CL sections can be seen in Figure 58.

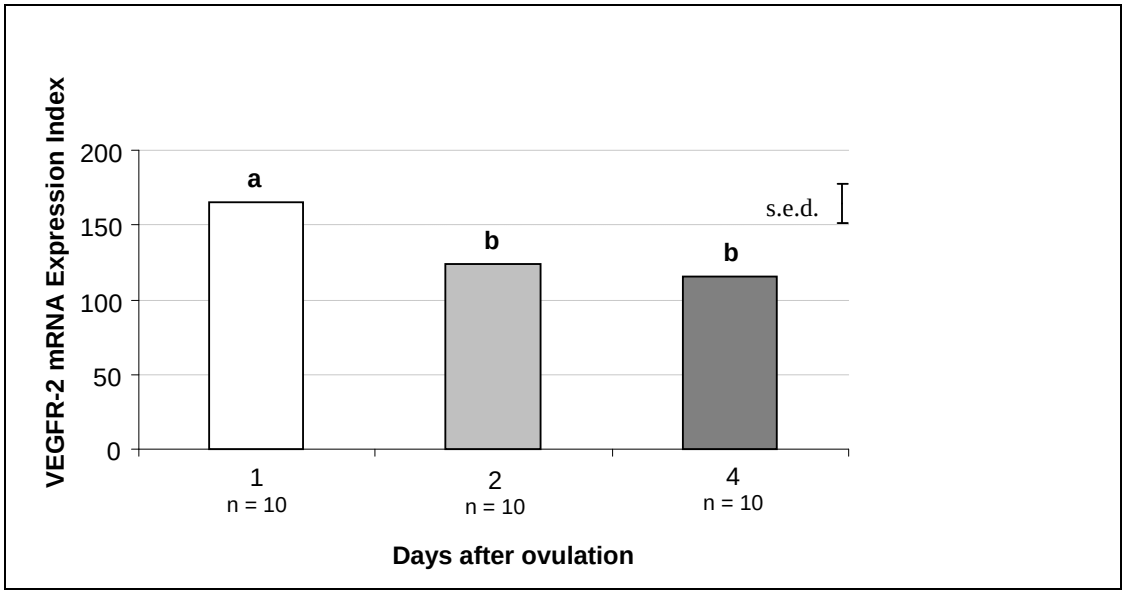

Figure 57. Mean Expression Index of VEGFR-2 mRNA observed 1, 2 and 4 days after ovulation in corpora lutea of seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 12.91, standard error of difference to compare mean values both within and between treatment groups. Different letters on bars indicate significant difference at 5% level. n = number of animals.

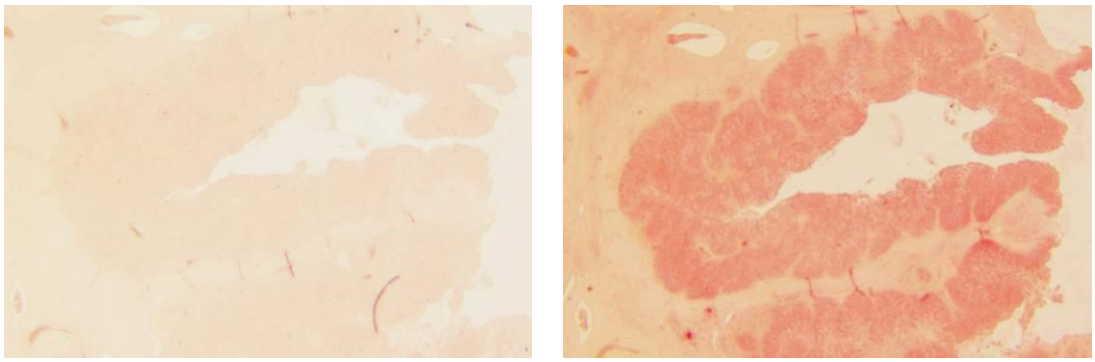

Figure 58. *In situ* hybridization showing the expression of VEGFR-2 mRNA in a 2 day *corpus luteum* from a progesterone primed seasonally anoestrous ewe induced to ovulate with small-dose multiple injections of GnRH. A sense image (left) is shown as negative control. Bright field microscopy, 50X magnification.

4.3.4 Angiopoietin-1 protein

A significant difference ($P<0.05$) was observed in Ang-1 protein levels between progesterone primed and non-primed animals. No significant difference ($P = 0.096$) was observed in Ang-1 protein levels between the different days after ovulation, however, a significant interaction ($P<0.05$) was observed between days and treatment.

Progesterone primed animals showed significantly higher ($P<0.05$) levels of Ang-1 at days 1 and 4 after ovulation than non-primed animals. However, no significant difference was observed between the two treatment groups at day 2 after ovulation. Progesterone primed animals maintained high levels of Ang-1 from day 1 up to day 4 after ovulation. Non-primed animals, on the other hand, showed a significant increase ($P<0.05$) in Ang-1 levels between day 1 and day 2 followed by a significant decrease ($P<0.05$) on day 4 after ovulation (Figure 59).

As expected, molecular weight for Ang-1 was around 55 kDa (Figure 60).

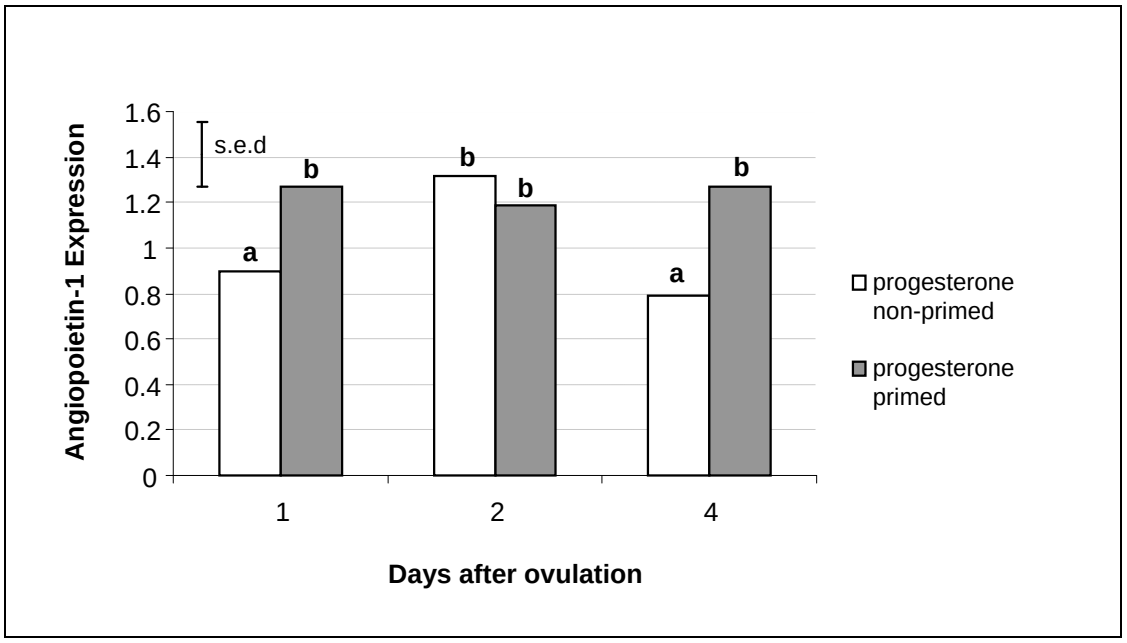

Figure 59. Mean expression of Angiopoietin-1 protein observed 1, 2 and 4 days after ovulation in corpora lutea of progesterone primed and non-primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 0.142, standard error of difference to compare mean values both within and between treatment groups. Different letters on bars indicate significant difference at 5% level. Number of animals per group per day = 5.

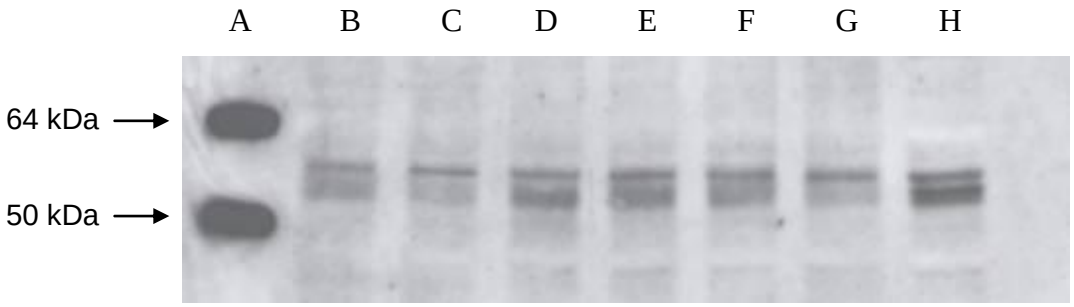

Figure 60. Representative autoradiograph of 10% acrylamide SDS/PAGE Angiopoietin-1 protein immunoblot showing luteal Angiopoietin-1 bands of early corpora lutea from progesterone primed and non-primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. Bands shown are of control sample (lane H) and luteal samples from 1 (lane B and C), 2 (lane D and E) and 4 (lane F and G) days after ovulation from progesterone primed and non-primed animals, respectively. Molecular weight markers (lane A) of 64 and 50 kDa are indicated by arrows.

4.3.5 Angiopoietin 1 mRNA

Ang-1 sense and anti-sense images of CL sections can be seen in Figure 61.

A significant difference ($P<0.05$) was observed in Ang-1 mRNA expression levels between progesterone primed and non-primed animals and between the different days after ovulation with no significant interaction ($P = 0.109$).

Progesterone primed animals showed significantly higher ($P<0.05$) overall luteal Ang-1 mRNA expression levels than non-primed animals (Figure 62). Ang-1 mRNA expression levels were significantly higher ($P>0.05$) on day 1 than on day 2 after ovulation with no significant difference being observed in Ang-1 mRNA expression levels between days 2 and 4 after ovulation (Figure 63).

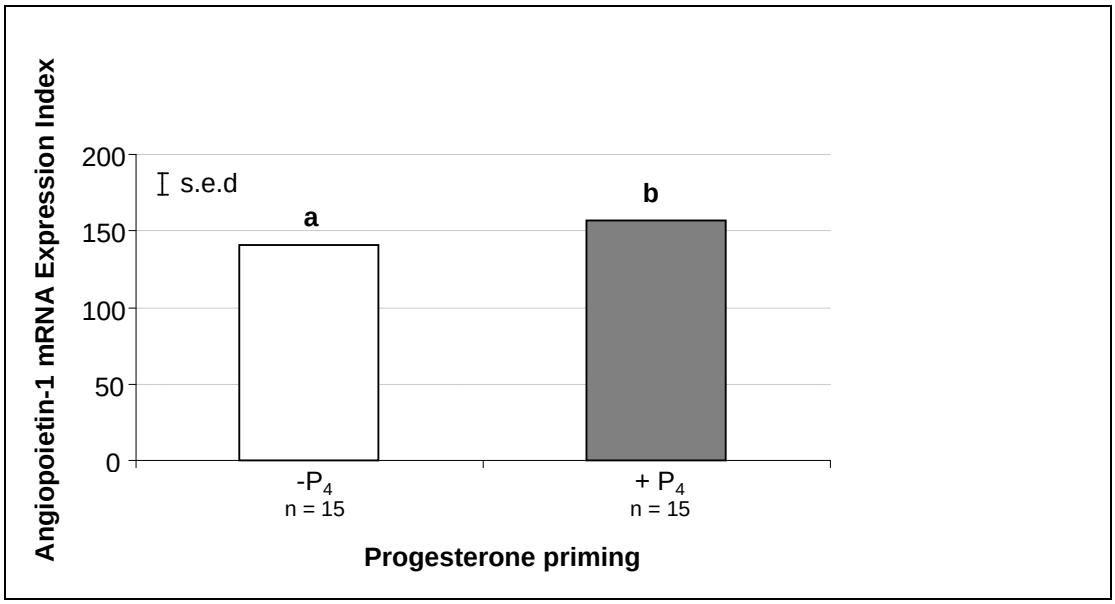

Figure 62. Mean Expression Index of Angiopoietin-1 mRNA observed in early corpora lutea of progesterone primed ($+P_4$) and non-primed ($-P_4$) seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 6.67, standard error of difference to compare mean values between treatment groups. Different letters on bars indicate significant difference at 5% level. n = number of animals.

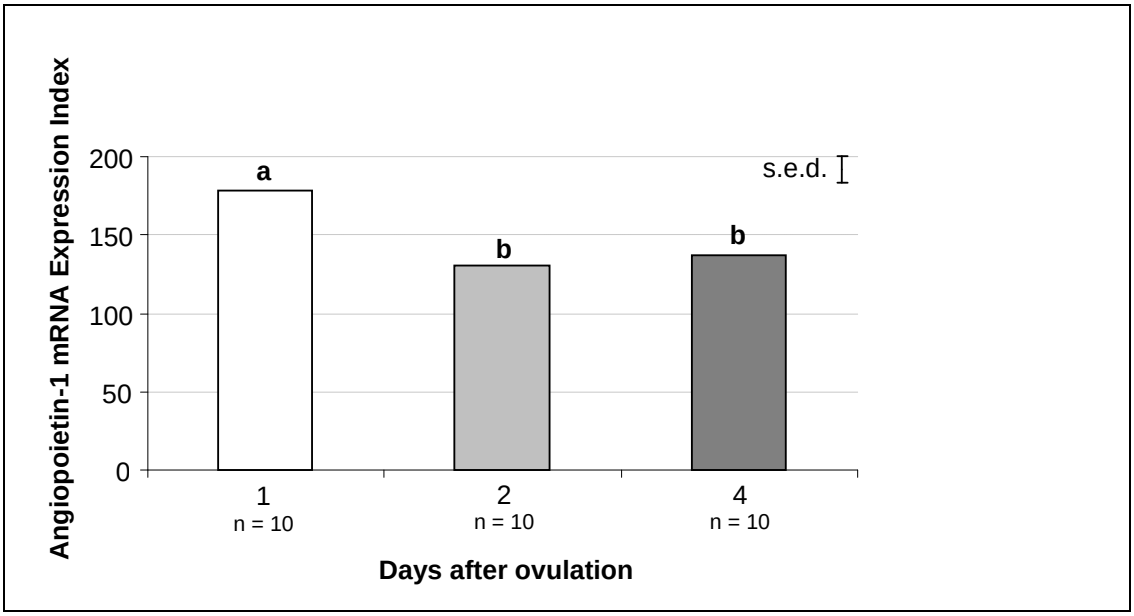

Figure 63. Mean Expression Index of Angiopoietin-1 mRNA observed 1, 2 and 4 days after ovulation in corpora lutea of seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 8.17, standard error of difference to compare mean values between days after ovulation. Different letters on bars indicate significant difference at 5% level. n = number of animals.

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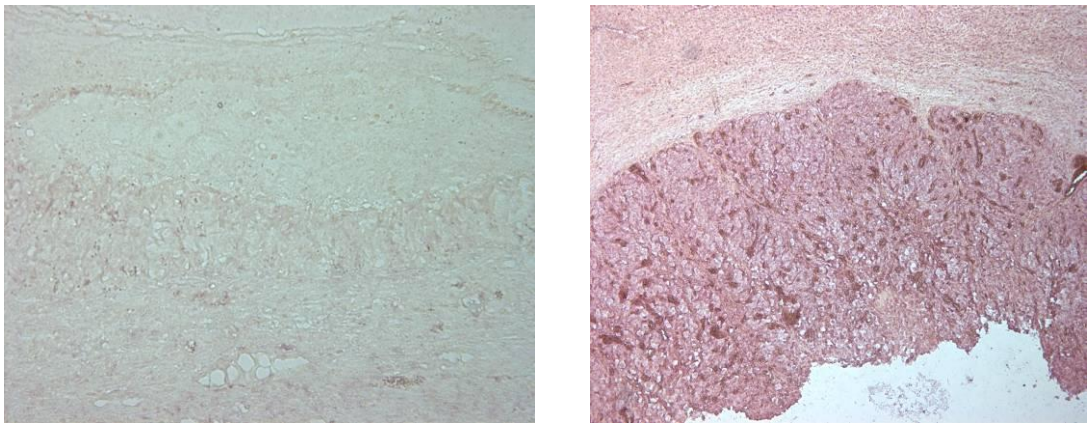

Figure 61. *In situ* hybridization showing the expression of Angiopoietin-1 mRNA in a 4-day *corpus luteum* from a progesterone primed seasonally anoestrous ewe induced to ovulate with small-dose multiple injections of GnRH. A sense image (left) is shown as negative control. Bright field microscopy, 100X magnification.

4.3.6 Angiopoietin-2 protein

No significant difference (P = 0.922) was observed in Ang-2 protein levels between progesterone primed and non-primed animals. A significant difference (P<0.05), however, was observed in Ang-2 protein levels between the different days after ovulation with no significant interaction (P = 0.109).

Ang-2 protein levels were significantly higher on day 1 after ovulation with no difference being observed in Ang-2 protein levels between days 2 and 4 after ovulation (Figure 62).

Angiopoietin-2 protein has a molecular weight of approximately 83 kDa. Luteal Ang-2 bands were observed, as expected, between 64 and 96 kDa (Figure 63).

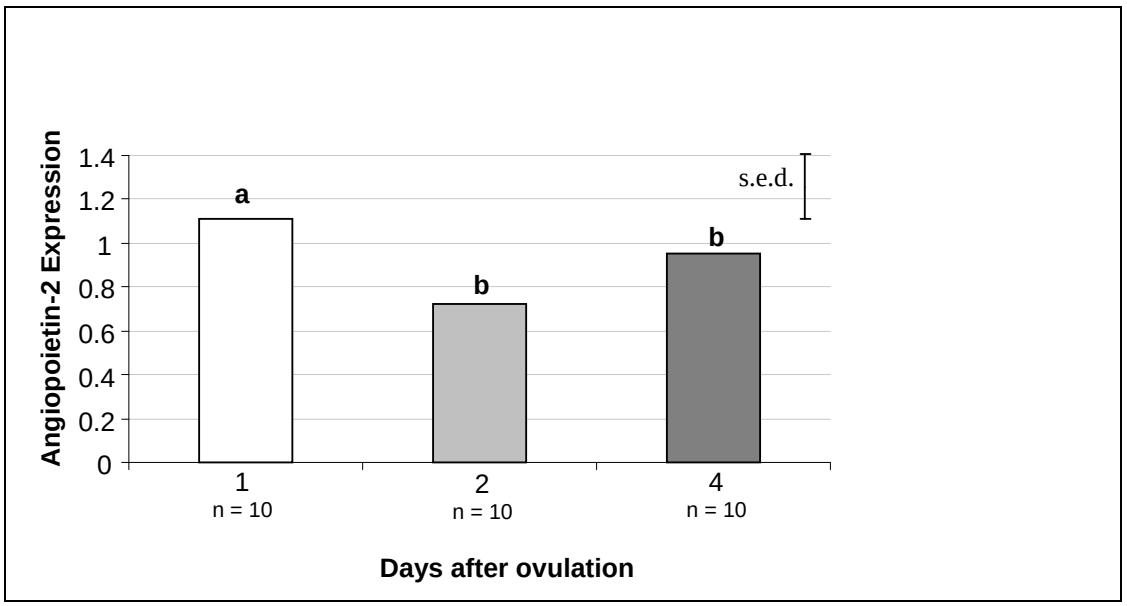

Figure 62. Mean expression of Angiopoietin-2 protein observed 1, 2 and 4 days after ovulation in corpora lutea of seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 0.151, standard error of difference to compare mean values between days after ovulation. Different letters on bars indicate significant difference at 5% level. n = number of animals.

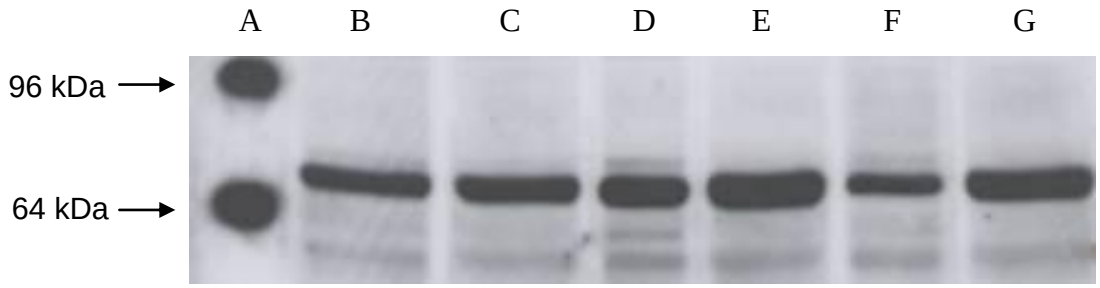

Figure 63. Representative autoradiograph of 10% acrylamide SDS/PAGE Angiopoietin-2 protein immunoblot showing luteal Angiopoietin-2 bands of early corpora lutea from progesterone primed and non-primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. Bands shown are of luteal samples from 1 (lanes B and C), 2 (lanes D and E) and 4 (lanes F and G) days after ovulation from progesterone primed and non-primed animals, respectively. Molecular weight markers (lane A) of 96 and 64 kDa are indicated by arrows.

4.3.7 Angiopoietin-2 mRNA

Ang-2 sense and anti-sense images of CL sections can be seen in Figure 64.

No significant difference ($P = 0.123$) was observed in Ang-2 mRNA expression levels between progesterone primed and non-primed animals. A significant difference ($P<0.05$) was observed in Ang-2 mRNA expression levels between the different days after ovulation with no significant interaction ($P = 0.352$).

Ang-2 mRNA expression levels were highest on day 1 but no significant difference was observed between days 2 and 4 after ovulation (Figure 65).

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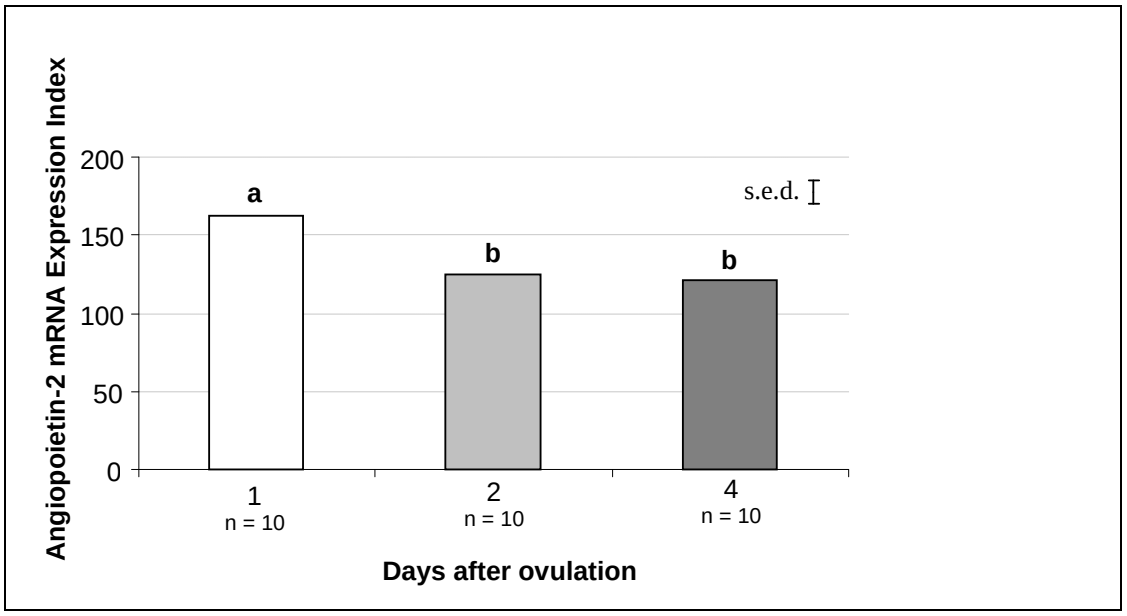

Figure 65. Mean Expression Index of Angiopoietin-2 mRNA observed 1, 2 and 4 days after ovulation in corpora lutea of seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 7.23, standard error of difference to compare mean values between days after ovulation. Different letters on bars indicate significant difference at 5% level. n = number of animals.

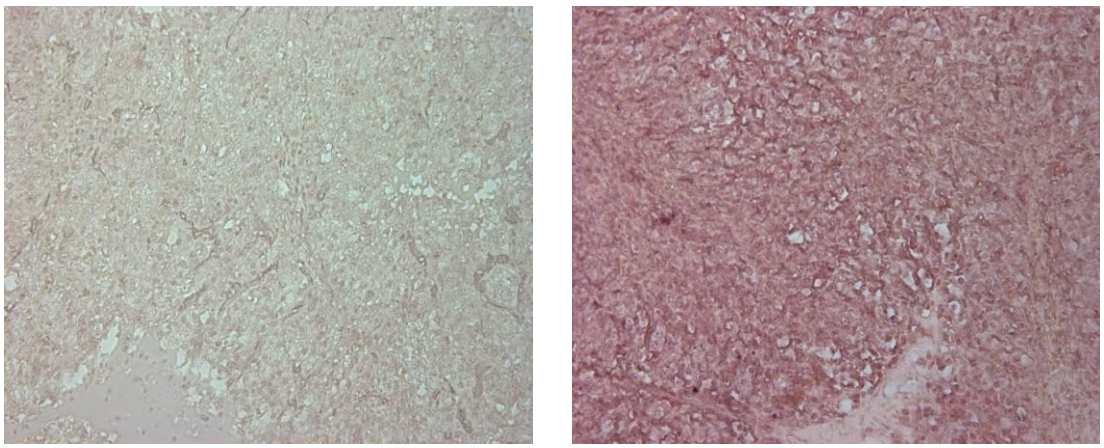

Figure 64. *In situ* hybridization showing the expression of Angiopoietin-2 mRNA in a 1-day *corpus luteum* from a progesterone primed seasonally anoestrous ewe induced to ovulate with small-dose multiple injections of GnRH. A sense image (left) is shown as negative control. Bright field microscopy, 200X magnification.

4.3.8 Angiopoietin-2: Angiopoietin-1 protein ratio

A significant difference (P<0.05) was observed in Ang-2:Ang-1 ratio between progesterone primed and non-primed animals as well as between the different days after ovulation but with no significant interaction (P = 0.250).

Non-primed animals showed significantly (P = 0.055) higher overall Ang-2:Ang-1 ratio during early CL development than progesterone primed animals (Figure 65).

The Ang-2:Ang-1 ratio significantly decreased (P<0.05) from day 1 to day 2 after ovulation and then significantly increased (P<0.05) by day 4 after ovulation. No significant difference was observed between the Ang-2:Ang-1 ratio on days 1 and 4 after ovulation (Figure 66).

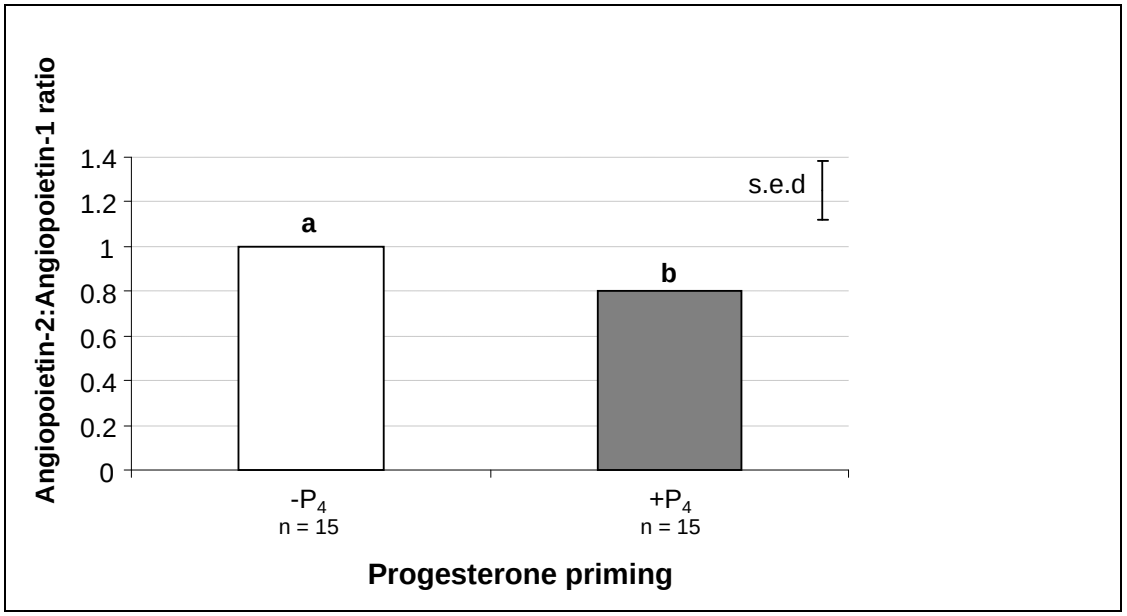

Figure 65. Mean Angiopoietin-2:Angiopoietin-1 ratio observed in early corpora lutea of progesterone primed ($+P_4$) and non-primed ($-P_4$)seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 0.136, standard error of difference to compare mean values between treatment groups. Different letters on bars indicate significant difference at 5% level. n = number of animals.

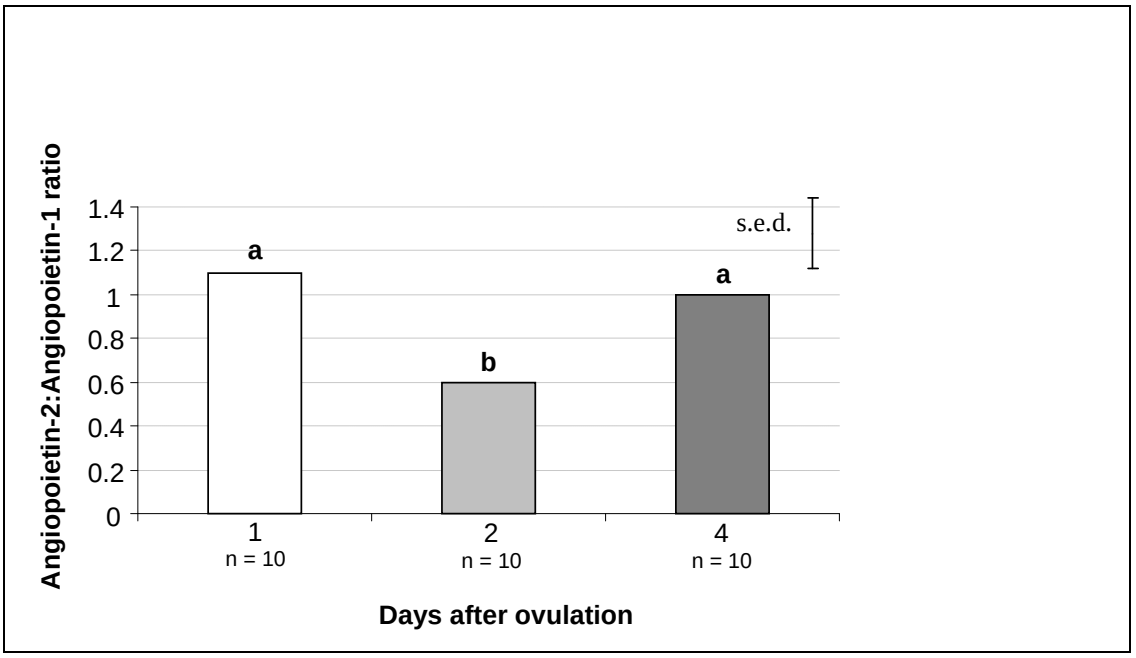

Figure 66. Mean Angiopoietin-2:Angiopoietin-1 protein ratio observed 1, 2 and 4 days after ovulation in corpora lutea of seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 0.160, standard error of difference to compare mean values between days after ovulation. Different letters on bars indicate significant difference at 5% level. n = number of animals.

4.3.9 TIE-2 protein

No significant difference (P = 0.265) was observed in TIE-2 protein levels between the two treatment groups or between different days after ovulation (P = 0.336). The treatment x day interaction was also not significant (P = 0.082).

TIE- 2 has a molecular weight of approximately 140 kDa. Luteal sample bands were observed between 96 and 148 kDa, as expected (Figure 67).

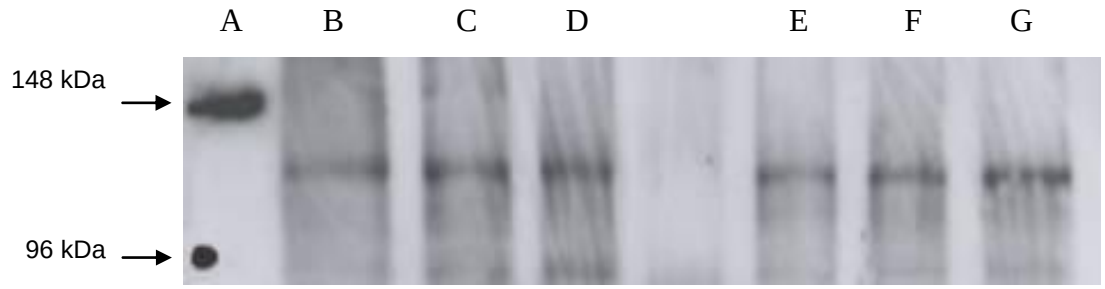

Figure 67. Representative autoradiograph of 10% acrylamide SDS/PAGE TIE-2 protein immunoblot showing luteal TIE-2 bands of early corpora lutea from progesterone primed and non-primed seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. Bands shown are of luteal samples from 1 (lanes B and E), 2 (lanes C and F) and 4 (lanes D and G) days after ovulation from progesterone primed and non-primed animals, respectively. Molecular weight markers (lane A) of 148 and 96 kDa are indicated by arrows.

4.3.10 TIE-2 mRNA

TIE-2 sense and anti-sense images of CL sections can be seen in Figure 68.

No significant difference ($P = 0.786$) was observed in TIE-2 mRNA expression levels between progesterone primed and non-primed animals. A significant difference ($P<0.05$) in TIE-2 mRNA expression levels was observed between the different days after ovulation with no significant interaction ($P = 0.527$).

TIE-2 mRNA expression levels were found to be significantly higher on day 1 than on day 2 and 4 after ovulation but with no significant difference being observed between days 2 and 4 after ovulation (Figure 69).

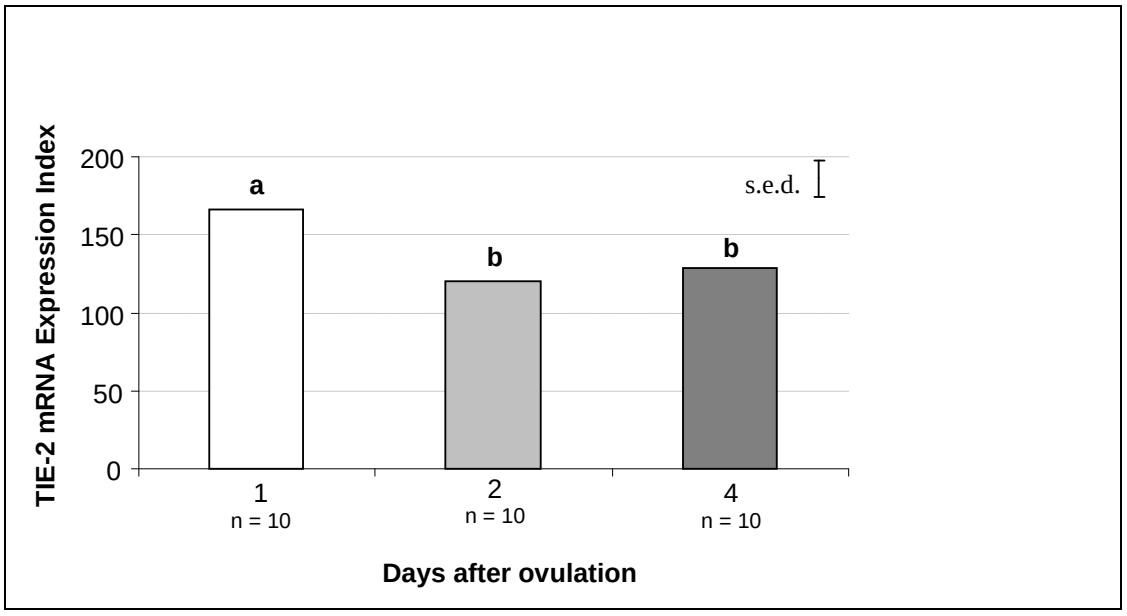

Figure 68. Mean Expression Index of TIE-2 mRNA observed 1, 2 and 4 days after ovulation in corpora lutea of seasonally anoestrous ewes induced to ovulate with small-dose multiple injections of GnRH. s.e.d. = 11.53, standard error of difference to compare mean values between days after ovulation. Different letters on bars indicate significant difference at 5% level. n = number of animals.

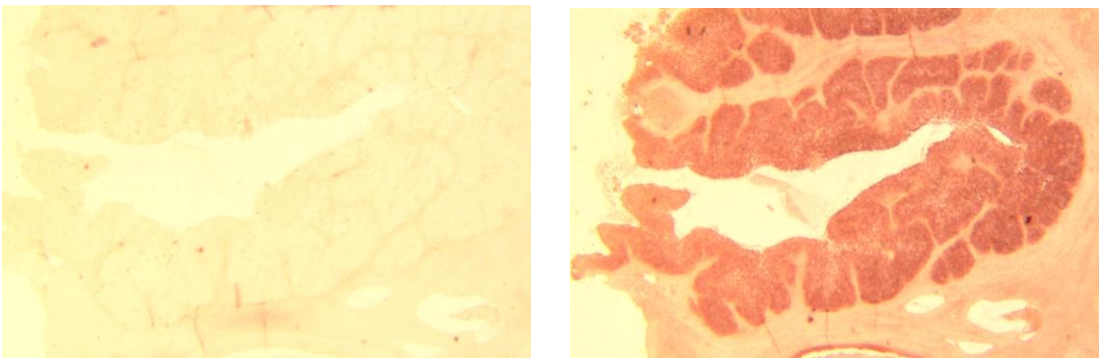

Figure 69. *In situ* hybridization showing the expression of TIE-2 mRNA in a 2-day *corpus luteum* from a progesterone primed seasonally anoestrous ewe induced to ovulate with small-dose multiple injections of GnRH. A sense image (left) is shown as negative control. Bright field microscopy, 50X magnification.

4.3.11 Von Willebrand Factor (VWF) protein

Von Willebrand Factor protein levels between primed and non-primed animals was very nearly significant (P = 0.062) with progesterone primed animals showing higher overall levels of VWF protein than non-primed ones. Although not significant (P = 0.083) a trend in VWF protein levels could be observed in the different days after ovulation with highest levels being observed on day 2 and lowest on day 4. No significant difference (P = 0.483) was observed in the interaction between days and treatment.

VWF distribution in luteal cells can be seen in Figure 70.

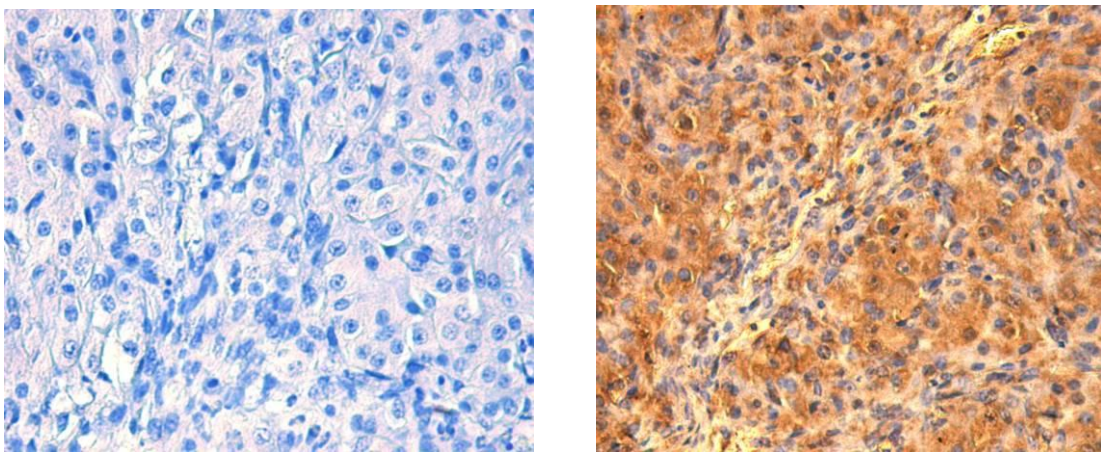

Figure 70. Immunohistochemistry showing the expression of VWF protein in a 1 day *corpus luteum* from a progesterone primed seasonally anoestrous ewe induced to ovulate with small-dose multiple injections of GnRH. A negative image (left) is shown as control. Bright field microscopy, 400X magnification.

Table 22

4.4 Discussion

The proportion of animals in this study that ovulated in response to small-dose multiple injections of GnRH are in agreement to those previously reported (Bramley et al., 2005, Haresign et al., 1996).

VEGF is a glycoprotein of approximately 45 kDa (Ferrara et al., 2003b) which dissociates into 22 kDa homodimers under reducing conditions (Redmer et al., 1996). The unexpected molecular weight observed for VEGF in the Western Blotting assays could be partially due to luteal VEGF being bound to heparin-containing proteins from the ECM. The different VEGF isoforms can be found either free or bound to the ECM (Ferrara, 2001, Poltorak et al., 1997, Wiesmann et al., 1997, Houck et al., 1991). The sheep CL has been shown to express at least three VEGF isoforms: $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (Schams and Berisha, 2004) with $VEGF_{121}$ corresponding to 1/3 of the total luteal VEGF content (Tamanini and De Ambrogi, 2004). As $VEGF_{121}$ is the only isoform found completely free in its diffusible form (Poltorak et al., 1997), the majority of the luteal VEGF must, therefore, be bound to heparin-containing proteogycans. If the VEGF present in the luteal samples was bound to other proteins this could explain its lower migration through the gel. The fact that heparinase treatment resulted in a band being present at 22 kDa supports this belief.

It has also been reported that some proteins, including glycoproteins, show anomalous behaviour during SDS/PAGE electrophoresis. Under appropriate conditions most reduced polypeptides bind to SDS in a constant weight ratio. However, many factors may interfere with the ability of proteins to form complexes with the SDS detergent, resulting in a different molecular weight than expected (Matagne et al., 1991). This could be another explanation for the higher molecular weight observed in the samples in this study. Although VEGF bands were observed at a much higher molecular weight than expected under reducing conditions, the specificity of the primary antibody to VEGF was confirmed by the blocking peptide assay and the bands observed in the membranes were proven to be those of VEGF.

Angiogenesis in the adult is mainly restricted to the female reproductive tract (Kaczmareck et al., 2005) and is regulated by ovarian angiogenic factors and their

receptors (Ferrara, 2001, Maisonpierre et al., 1997, Reynolds et al., 2000). VEGF and Angiopoietins play an essential role throughout luteal development stimulating the proliferation, maturation and finally regression of blood vessels (Benjamin et al., 1998, Ferrara et al., 1992, Davis et al., 1996, Maisonpierre et al., 1997).

As the ovulatory follicle ruptures, theca vessels invade the granulosa layer and new blood vessels are formed to support the developing CL (Shimizu et al., 2007). Adequate blood supply is essential for normal tissue growth and development (Tamanini and De Ambrogi, 2004) and the developing CL requires an extensive vascular network to be established to support the morphological and functional changes taking place soon after ovulation (Schams and Berisha, 2004, Al-zi'abi et al., 2003).

GnRH-induced ovulation in seasonally anoestrous ewes, in the absence of progesterone, results in the development of a defective CL (McLeod et al., 1982b, Southee et al., 1988b). As inadequate vascularisation has been proposed as a possible cause of defective luteal function (Reynolds and Redmer, 1998), in this experiment we tested the hypothesis that progesterone priming stimulates angiogenesis by altering the expression and production of angiogenic factors and their receptors in the newly developing CL immediately after ovulation.

By using seasonally anoestrous ewes primed and non-primed with progesterone prior to induced ovulation by small-dose multiple injections of GnRH, we were able to study the differences in angiogenic factor levels between functional and defective CL at their early stage of development.

Results of this study have shown that the lack of progesterone priming prior to GnRH-induced ovulation has a negative impact on the VEGF-Angiopoietin system within the developing CL, probably compromising luteal angiogenesis and thus luteal function.

VEGF levels, during early luteal development are expected to increase soon after ovulation to stimulate blood vessel formation. These levels gradually decrease by the mid-luteal phase when the CL has achieved its maximum size. During the mid-luteal phase cell proliferation is reduced and the vascular bed formed soon after ovulation matures and stabilizes (Ribeiro et al., 2006, Tamanini and De Ambrogi, 2004, Kaczmareck et al., 2005).

CL from progesterone primed and non-primed ewes induced to ovulate with GnRH showed low levels of VEGF straight after ovulation, followed a significant increase by day 2 after ovulation. Although VEGF levels are reported to be high during early luteal development (Redmer et al., 1996), it has been recently reported that luteal VEGF levels are low immediately after ovulation (Fraser and Duncan, 2009), as observed in this study on day 1 CL. The increase in VEGF levels 2 days after ovulation is in agreement with previous studies on VEGF expression during early luteal development (Kaczmareck et al., 2005, Al-zi'abi et al., 2003).

Although no significant difference was observed in luteal VEGF levels between progesterone primed and non-primed animals on day 1 and 4 after ovulation, levels were significantly lower in non-primed compared with progesterone primed animals on day 2 with VEGF levels in CL from non-primed ewes being as low as those of progesterone primed animals on day 4 after ovulation. These findings indicate that CL from non-primed animals produce lower levels of VEGF at a critical time for angiogenesis in early luteal development. These lower levels of VEGF could lead to the formation of a limited vascular network which might prove to be inadequate to support normal luteal function.

VEGFR-2 levels in CL on day 1 after ovulation were not different between progesterone primed and non-primed animals. While VEGFR-2 levels in CL from progesterone primed animals remained constant from day 1 up to day 4 after ovulation, confirming earlier findings of a similar pattern as that of VEGF protein (Tamanini and De Ambrogi, 2004), CL from non-primed animals, on the other hand, showed a marked and significant decrease in VEGFR-2 levels by days 2 and 4 after ovulation. These low VEGFR-2 levels found in the CL of non-primed ewes, at a time when angiogenesis should be high, further aggravate the low VEGF levels observed on day 2 after ovulation. These findings suggest that at a time when both VEGF and VEGFR-2 are expected to be high, CL from non-primed animals show significantly lower levels of both VEGF protein and its receptor than those from progesterone primed animals. These findings strongly support the idea that a defective stimulus for the formation of new blood vessels could result in inadequate luteal vascularity and thus be the cause of defective luteal function.

Overall VEGFR-2 mRNA expression, however, did not vary between primed and non-primed animals. VEGFR-2 mRNA levels were highest soon after ovulation followed by a significant decrease from day 1 to day 2 which persisted until day 4 after ovulation. These reports are in agreement with previously reported data in humans in which VEGFR-2 mRNA levels were found to be high soon after ovulation (Ferrara et al., 2003a) followed by a significant decrease in expression as the luteal phase progressed (Ribeiro et al., 2006). Lack of a difference in the VEGFR-2 mRNA mRNA expression and a presence of difference in the protein levels in the CL between progesterone and non-primed ewes may suggest the effect of progesterone priming at the translational level for VEGFR-2.

The Angiopoietin-TIE system is essential for functional luteal development and work in close association with the VEGF system to regulate ovarian angiogenesis (Shimizu et al., 2007). Ang-1 levels are mainly associated with vascular invasion and maturation while Ang-2 levels play an important role in vessel formation and regression (Maisonpierre et al., 1997). During early CL development Ang-2 levels are high and decrease by the mid-luteal phase. Ang-1 levels are low soon after ovulation and increase as the CL matures (Schams and Berisha, 2004, Sugino et al., 2005).

In this study, Ang-1 level was constant and high in CL from progesterone primed animals from day 1 to day 4 after ovulation. CL from non-primed animals, on the other hand, had significantly lower Ang-1 levels on day 1, showed a significant increase by day 2 (but not higher than primed CL) followed by a significant decrease by day 4 after ovulation. Ang-1 is known to stimulate vascular invasion (Maisonpierre et al., 1997) and these low levels of Ang-1, observed on day 1 CL from non-primed animals, may compromise the vascular invasion taking place soon after ovulation. This could result in a limited and insufficient vascular bed being developed in the CL from non-primed animals.

Although Ang-1 levels were similar between primed and non-primed animals on day 2, on day 4 CL from non-primed animals showed significantly lower Ang-1 levels than those of progesterone primed animals. At a time when blood vessels should be starting to stabilize, low levels of Ang-1 may indicate immature vascularity. Therefore, besides lower vascular invasion on day 1 and lower vascular

density due to low VEGF and VEGFR-2 levels on day 2, CL from non-primed animals seem unable to induce a desirable level of maturation on day 4 after ovulation on those blood vessels that have been formed. Non-stable vessels tend to regress as VEGF levels start to decline (Hayashi et al., 2003). Therefore, instead of developing a mature and stable vascularity by the mid-luteal phase, defective CL may well show early signs of vascular regression as a consequence of these differences in Ang-1 protein levels.

Overall Ang-1 mRNA expression was significantly higher in CL from progesterone primed than non-primed animals which closely reflects the Ang-1 protein production by the luteal cells.

No significant difference was observed in Ang-2 levels between CL from primed and non-primed animals or during the different days after ovulation. These findings are in agreement with previously reported data in which Ang-2 levels remain stable throughout luteal development (Schams and Berisha, 2004). Interestingly, there was a significant decrease in Ang-2 mRNA expression by day 2 and 4 after ovulation, however, no significant difference was observed between progesterone primed and non-primed animals.

Ang-2:Ang-1 ratio, also referred to as the stabilization ratio, in conjunction with VEGF levels will determine whether new blood vessels are to be formed, matured or regress (Hayashi et al., 2003). CL from non-primed animals showed significantly higher levels of Ang-2:Ang-1 ratio during early luteal development. Furthermore, the Ang-2:Ang-1 ratio was highest on days 1 and 4 after ovulation. These high levels of Ang-2:Ang-1 ratio observed soon after ovulation are in agreement with previously reported data for functional CL (Schams and Berisha, 2004, Sugino et al., 2005). However, low levels of VEGF and VEGFR-2 associated with high Ang-2:Ang-1 ratio observed on day 4 in CL from non-primed animals indicate a tendency of the newly formed blood vessels to regress instead of maturing as expected.

TIE-2 protein and mRNA expression levels did not vary significantly between treatment groups. However, TIE-2 mRNA showed a significant decrease from day 1 to 2 after ovulation with no significant variation on luteal TIE-2 protein levels.

Higher Ang-2 compared to Ang-1 levels on day 4 are likely to result in de-stabilization of blood vessels in CL from non-primed animals. As Ang-2 binds to

TIE-2 with the same affinity as Ang-1, high Ang-2 levels mean less receptor available for Ang-1-induced blood vessel maturation. Furthermore, the low levels of VEGF and VEGFR-2 result in the degeneration of the already limited newly formed vasculature in the early CL of non-primed animals, at a time when functional CL vessel network should be starting to stabilize to sustain the developing CL.

Von Willebrand Factor (VWF) levels showed no significant difference between defective and functional CLs at any time of luteal development. However, differences between defective and functional CL were very nearly significant with progesterone primed animals showing higher overall levels of VWF protein than non-primed animals during early luteal development, supporting the hypothesis that progesterone priming ensures normal luteal function by enabling greater vascular development to take place during the early luteal phase. Furthermore, a trend could be observed in VWF protein levels in the different days after ovulation with the highest levels being observed on day 2 and the lowest on day 4. These findings are in agreement with the levels of the other angiogenic factors observed on those days, supporting the belief that on day 2 angiogenesis is at its highest whilst by day 4 neovascularisation is declining and vascular maturation starting to take place. The levels of angiogenic factors observed on day 4 after ovulation suggest that CL from primed animals show greater stabilization of blood vessels than those from non-primed animals. This seems to be true as CL from non-primed animals show significant lower vascular surface area by day 12 after ovulation than those from progesterone primed animals (Bramley et al., 2005).

A lack of significant difference in the levels of some angiogenic factors between progesterone primed and non-primed animals when it was expected to be significant (according to our hypothesis) can also be supported by the fact that CL from non-primed animals were of mixed type (some are defective and others are functional) where CL from primed groups were normal only (Southee et al., 1988b, Haresign et al., 1996, McLeod et al., 1982b). This may give more variation in the data from non-primed animals and may therefore mask the differences between the two treatment groups. Furthermore, low number of animals (n = 4 or 5) per treatment group aggravates the lack of difference; had there been higher number of animals more obvious differences between treatments could have been observed.

In conclusion, the lack of progesterone priming seems to have a great impact on VEGF, VEGFR-2, Ang-1 and VWF protein production. A disruption in the delicate balance that governs angiogenesis may lead to inadequate vascular support by the mid-luteal phase and thus result in defective luteal function. Furthermore, low vascularity leads to lower levels of nutrients, hormones and steroidal precursors being delivered to the luteal cells. Low LH and GH being delivered to the CL consequently results in low progesterone output by the small and large luteal cells, a characteristic previously described for defective CL. Low progesterone levels may also compromise oxytocin receptor blocking in the endometrium and thus lead to premature PGF-2α secretion and consequently early luteal regression.

CHAPTER 5

Summary and Conclusions

5.1 Summary of Results

Infertility is a serious issue in both human and animal medicine and has a major economic impact on livestock production (Campbell et al., 2003). Many factors have been attributed as causes of infertility, among them defective luteal function (Yaniz et al., 2008). Defective luteal function is a relatively common occurrence in livestock reproduction. Seasonal species such as sheep, goats and mares show an initial short cycle during the transition from anoestrus to sexual activity, with a CL that prematurely regresses 5 to 6 days after ovulation (Rosa and Bryant, 2003). Non-seasonal animals such as cows are similarly affected. The first post-partum ovulation in both dairy and beef cows is generally silent, followed by a short cycle in more than 70% of the animals (Crowe, 2008).

It is now widely accepted that pulsatile GnRH treatment of seasonally anoestrous ewes is capable of inducing the final stages of preovulatory follicle development and ovulation in most animals (McLeod et al., 1982b, McLeod et al., 1982a, McLeod et al., 1983); however, a defective CL is formed in the vast majority (60-80%) of animals. Progesterone priming prior to GnRH treatment results in normal luteal function (Southee et al., 1988b, Haresign et al., 1996, Hunter et al., 1988, Husein and Kriddli, 2003). Consequently, seasonally anoestrous ewes treated with pulsatile injections of GnRH, with and without progesterone priming, provides a useful and controlled *in vivo* model in which to study defective luteal function.

The defective CL that originates after GnRH-induced ovulation in the absence of progesterone priming is basically characterized by low progesterone production and early regression (Southee et al., 1988b). Although capable of producing normal levels of progesterone over the first 4 or 5 days after ovulation, progesterone returns to basal levels by day 6 after ovulation (Hunter et al., 1988). Morphological differences between functional and defective CL become evident 5 days after ovulation when defective CL start showing signs of early regression (Hunter et al., 1989).

The exact mechanisms by which progesterone priming prior to GnRH treatment guarantees normal luteal function are still unclear. It has been suggested that inadequate priming of the pre-ovulatory follicle by oestrogen results in abnormal

luteinisation of the granulosa cells which will compose the steroidogenic parenchyma of the developing CL (Murdoch and Van Kirk, 1998) resulting in low progesterone output. Another concept is that inadequate development of the follicular microvasculature leads to the formation of a luteal vascular network unable to support the demands of the developing CL (Southee et al., 1988a).

Normal follicle development and ovulation in cyclic animals directly depends upon adequate vascularisation (Shimizu et al., 2007). The thecal vascular network is the main source of nutrients, gonadotrophins and steroid precursors to the developing follicle (Mattioli et al., 2001, Kaczmareck et al., 2005). Around the time of the LH surge, the theca capillaries invade and sprout into the granulosa layer to form a dense vascular network (Shimizu et al., 2007). After the LH surge the theca and granulosa layer undergo dramatic changes (luteinisation) to form the secretory parenchyma of the CL (Schams and Berisha, 2004, Berisha and Schams, 2005).

In order to meet the demands of the rapid changes taking place in the early CL a well established vascular supply is essential (Robinson et al., 2007). The high proliferation of endothelial cells in the early luteal phase is responsible for the increased vascularity of mature CL. Besides, the presence of a dense vascular network is essential for optimal delivery of nutrients and progesterone precursors to the developing CL (Schams and Berisha, 2004, Al-zi'abi et al., 2003). Therefore, an adequate vascular supply seems to be closely associated with progesterone production and normal luteal function.

Many factors have been considered as positive regulators of angiogenesis, including the VEGF, the Angiopoietins and their receptors (Bramley et al., 2005, Ferrara et al., 2003b, Kaczmareck et al., 2005). The main roles of VEGF in angiogenesis consist of stimulating endothelial cell proliferation and migration as well as maintaining immature blood vessel viability for either neovascularisation or regression (Benjamin et al., 1998). VEGFR-2 is the key signalling receptor for VEGF (Ferrara, 2001) and is indispensible for angiogenesis to take place (Gille et al., 2001).

Unlike VEGF, the Angiopoietins are mainly related to blood vessel stabilization. Ang-1 regulates vascular maturation after the initial stages of blood vessel formation (Davis et al., 1996) while Ang-2 destabilizes existing vessels by

loosening their supporting cell matrix (Tamanini and De Ambrogi, 2004). Both Angiopoietins bind with the same affinity to TIE-2 receptor (Suri et al., 1996), however, Ang-2 is unable to cause TIE-2 phosphorylation thus antagonizing Ang-1 activity mainly by competing for the same receptor (Maisonpierre et al., 1997).

Although individual angiogenic factors are important, angiogenesis depends on the interaction between these factors (Tamanini and De Ambrogi, 2004). When both the Ang-2:Ang-1 ratio and VEGF levels are high angiogenesis results in the formation of new blood vessels, however, when Ang-2:Ang-1 ratio is high and VEGF levels are low vascular regression can be seen. Furthermore, low Ang-2:Ang-1 ratio usually results in the stabilization of blood vessels (Hayashi et al., 2003).

Follicular and luteal growth are directly dependent on blood vessel formation and proliferation (Ferrara et al., 2003a) and therefore dependent on VEGF (Ferrara, 2001, Dickson et al., 2001, Hunter et al., 2004, Wulff et al., 2001b) and the Angiopoietin system (Tait and Jones, 2004, Maisonpierre et al., 1997).

As progesterone priming prior to GnRH treatment overcomes defective luteal functionality in seasonally anoestrous ewes, and as normal luteal function seems to be closely related to ovarian vascularity, it led us to the hypothesis that progesterone priming might be involved in regulating angiogenic factor levels in the pre-ovulatory follicle and early CL. This hypothesis was tested using the GnRH treated seasonally anoestrous ewe, with and without progesterone priming as an experimental model. Animals were slaughtered at various times before and after the expected time of ovulation to permit comparison of both ovarian follicles prior to ovulation and early developing CL after ovulation.

In this study it was observed that neither progesterone priming nor the LH surge had any significant overall influence over VEGF, VEGFR-2, Ang-1, Ang-2 or TIE-2 protein or mRNA levels in small follicles. However, VEGF levels were found to be significantly lower in both treatment groups after the LH surge. These low levels of VEGF, associated with unaltered Angiopoietin levels, suggest that vascular development in these follicles had either stopped or started to regress. Degradation of the vascular network would result in decreased nutrients reaching the follicle leading to atresia (Tamanini and De Ambrogi, 2004). All small follicles analysed in this study were smaller than 3.5 mm in diameter and may have either none or very

limited number of receptors for LH in the granulosa layer (Hunter et al., 2004). Therefore, after the LH surge, these follicles were expected to either be dormant, if they were not part of the cohort of follicles selected for development, or undergoing atresia if they were (Driancourt, 2001).

The large potential pre-ovulatory follicles, on the other hand, showed a very different response to progesterone priming and LH surge. Non-primed animals showed significantly lower levels of VEGF than progesterone primed ones. Furthermore, non-primed animals showed significantly lower levels of VEGFR-2, Ang-1 and Ang-2 protein after the LH surge but no difference in TIE-2 levels. At a time when angiogenesis is expected to be high and theca blood vessels are invading and sprouting into the granulosa layer (Ferrara et al., 1992), low VEGF and VEGFR-2 associated to low Ang-2 levels could lead to poor sprouting of the theca capillaries and deficient neovascularisation of the CL that forms from these pre-ovulatory follicles.

As the formation of new blood vessels during luteal development originates from the existing vascular network of the follicle (Kaczmareck et al., 2005, Berisha and Schams, 2005, Zheng et al., 1993), low levels of angiogenic factors after the LH surge not only interfere with follicular irrigation but may also compromise luteal vascularity and consequently luteal function. Furthermore, Ang-1 and Ang-2 after the LH surge are known to influence luteinisation of the follicular layers (Hayashi et al., 2003). The lower levels of both Angiopoietins observed in non-primed animals after the LH surge would therefore likely interfere with the luteinisation process and progesterone production, further aggravating the defective luteal function (Murdoch and Van Kirk, 1998).

The effects of progesterone priming prior to GnRH treatment were also analysed in CL at 1, 2 and 4 days after ovulation, before morphological differences between defective and functional CLs became apparent (Hunter et al., 1988, Southee et al., 1988b).

Although no significant difference was observed in VEGF levels between treatment groups on day 1 and 4, non-primed animals showed significantly lower levels of VEGF than primed animals on day 2 after ovulation. Furthermore, non-primed animals showed significantly lower levels of VEGFR-2 on days 2 and 4 after

ovulation. Non-primed animals also expressed significantly lower levels of Ang-1 on day 4 after ovulation than primed animals. Progesterone priming, however, had no significant effect on Ang-2 levels during early luteal development.

The lower VEGF and VEGFR-2 observed in non-primed animals on day 2 after ovulation may result in limited angiogenesis and/or neovascularisation taking place in the developing CL. Considering that these new blood vessels are being formed from sprouting of the pre-existent luteal vasculature and that this initial luteal vascular network seems to be much more limited in non-primed animals due to the events that took place during follicular development after the LH surge, it is reasonable to believe that these lower levels of VEGF and VEGFR-2 during early luteal development may have a serious impact in restricting the luteal vascular network being formed. Although sufficient to support the CL during its early stages of development, this limited vascular supply may not be enough to support the demands of the growing CL and this may be the reason that at 5 days after ovulation morphological differences start to appear in the CL between non-primed (defective CL) and primed animals (functional CL).

Von Willebrand Factor data has shown that progesterone primed animals have greater vascular area than non-primed ones during early luteal development. Although this difference failed to reach the 5% statistical significance (P=0.062) its biological significance cannot be overlooked. This poorer vascular coverage observed in defective CL when compared to functional CL may not only interfere with early luteal development but may also prove to be a poorer template for any further vascularisation taking place during the luteal phase and may thus account for the reduced vascular surface observed in defective CL later in luteal development (Bramley et al., 2005). It is important to consider that approximately ¼ of the CL from non-primed animals may show normal luteal function (McLeod et al., 1982b, Haresign et al., 1996, Southee et al., 1988b) which is likely to have contributed to the lack of statistical difference in the luteal vascular area between primed and non-primed animals observed in this study.

The lower Ang-1 levels observed in non-primed animals on day 4 after ovulation suggest a lack of maturation and/or destabilisation of the blood vessels present. Higher Ang-2 compared to Ang-1 levels on day 4 result in less TIE-2

receptors being available for binding with the already limited amount of Ang-1 present. Moreover, a higher Ang2:Ang1 ratio at this time may cause the destabilisation of the existing newly formed blood vessels. In addition, the relatively low levels of VEGF and VEGFR-2 observed on day 4 after ovulation in non-primed animals may also aggravate the process of degeneration of the newly formed vasculature whereas in progesterone-primed animals a lower Ang2:Ang1 ratio along with comparatively higher levels of VEGF and VEGFR-2 ensures further development and stabilisation of the existing vascular network and hence a functional CL.

5.2 Conclusions

It is concluded that the lack of progesterone priming had no influence on VEGF, VEGFR-2, Ang-1, Ang-2 and TIE-2 protein or mRNA expression levels in small follicles of seasonally anoestrous ewes treated with GnRH. However, the lack of progesterone priming resulted in lower levels of VEGF, VEGFR-2, Ang-1 and Ang-2 protein after the LH surge in large pre-ovulatory follicles. No change was observed in Ang-1 nor Ang-2 mRNA or in TIE-2 protein or mRNA expression levels in large follicles.

In the early CL lack of progesterone priming compromises the production of all angiogenic factors, with the exception of Ang-2 resulting in reduced vascular area. Furthermore, progesterone priming seems to influence the translation and post-translation processes in VEGFR-2 and Ang-1 production by early CL.

The limited sprouting and blood vessel formation and the compromised luteinisation taking place in large pre-ovulatory follicles after the LH surge, associated with low neovascularisation by day 2 and vascular instability and regression by day 4 after ovulation, could possibly explain the defective luteal function observed in non-primed seasonally anoestrous ewes treated with GnRH.

Although angiogenesis plays a major role in luteal functionality, defective luteal function is most likely caused by an association of events taking place in late follicular and early luteal development, with defective production of angiogenic factors being one of these many events.

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