Progesterone exposure of seasonally anoestrous ewes alters the expression of angiogenic growth factors in preovulatory follicles

A.C.M. Christensen a,b, W. Haresign a, M. Khalid b,∗

a Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Penglais Campus, Aberystwyth, Ceredigion, UK
b Department of Production and Population Health, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire, UK

Abstract
Small-dose, multiple injections of GnRH given to seasonally anoestrous ewes induce final stages of the preovulatory follicle development, but result in an high incidence of defective CL unless animals are primed with progesterone, which completely eliminates luteal dysfunction. Progesterone priming upregulates luteal vascularization; however, its effect on follicular angiogenesis is poorly understood. This study tested the hypothesis that progesterone priming of seasonally anoestrous ewes treated with dose multiple injections of GnRH eliminates defective luteal function by altering the expression of vascular endothelial growth factor (VEGF), VEGF receptor-2, angiopoietin (ANG)-1, ANG-2, and TIE-2 during early and late preovulatory follicle development. Ten seasonally anoestrous ewes were given 20 mg of progesterone im 3 days before the start of GnRH treatment; 10 other animals served as controls. Intravenous injections of 500 ng GnRH were given to all animals every 2 hours for 28 hours, followed at 30 hours with a 300-μg GnRH bolus injection to synchronize the preovulatory LH surge. Ovaries were collected at 24 and 46 hours after the start of GnRH treatment. Small (2–2.5 mm) and large (>2.5 mm) follicles were analyzed for protein and mRNA expression of the angiogenic factors using immunohistochemistry and in situ hybridization assays. Progesterone priming did not have an influence on angiogenic factor levels in small follicles. However, progesterone-primed animals showed significantly (P < 0.05) higher levels of VEGF, VEGFR-2, ANG-1, and ANG-2 in large follicles compared with nonprimed ones. These data suggest that progesterone priming alters the expression of angiogenic factors in large preovulatory follicles, ensuring adequate luteal development and function.

1. Introduction
Infertility is a major issue in animal and human medicine [1], with defective luteal function being attributed as one of its significant causes [2]. Luteal dysfunction is a relatively common occurrence in livestock reproduction at the beginning of puberty or post-partum [3,4] and seasonal [5] anoestrus. Ovulation of a healthy and mature follicle is paramount to normal luteal development and function. The lack of understanding of the factors involved in follicular development and maturation pose limitations to the development of successful fertility protocols. Seasonally anoestrous ewes induced to ovulate with repeated small doses of GnRH have been widely used as a model to study luteal function as it drives the final stages of preovulatory follicle development leading to the induction of ovulation [6–10]. However, inadequate luteal function is present in the vast majority of these animals unless they have been primed with progesterone before GnRH therapy [8,9]. This pattern of defective luteal function has been
attributed to inadequate follicular development before ovulation [7–9,11].

It has been suggested that the influence of progesterone on subsequent luteal function starts early in follicular development, affecting dominant follicle characteristics and development before ovulation because priming seasonally anestrous ewes with progesterone 3 to 5 days before the start of GnRH treatment ensures normal luteal function in majority of the animals [8,12]. However, evidence to support such suggestions has remained elusive.

To support the increasing blood flow during follicular development, the theca capillaries undergo extensive angiogenesis [13], mainly regulated by the members of the vascular endothelial growth factor (VEGF) and the angiopoietin (ANG) family [14–17]. Vascular endothelial growth factor is essential for follicular development and function because it stimulates endothelial cell proliferation and migration, maintains immature blood vessel viability [18], and ensures endothelial cell survival through the phosphorylation of its main receptor VEGF receptor (R)-2 (Flk-1) [19]. After the initial stages of vascular development, ANG-1 is responsible for vascular stabilization and maturation [20], whereas ANG-2 is involved in destabilization of existing vessels [15]. Both ANG-1 and -2 bind to the TIE-2 receptor with similar affinity and thus act as antagonists by competing for the same receptor [21]. The ANG-2:ANG-1 ratio is used as an index of vascular stabilization in conjunction with VEGF levels [22]. When the ANG-2:ANG-1 ratio and VEGF levels are high, new blood vessel networks are formed. However, when the ANG-2:ANG-1 ratio is low but VEGF levels are low, regression of blood vessels happens and a low ANG-2:ANG-1 ratio along with relatively low levels of VEGF result in stabilization of blood vessels [23].

Because vascularity and normal ovarian function are closely related [16,17,24], it is a possibility that inadequate development of the microvasculature of the preovulatory follicle may be the underlying cause of defective luteal function in nonprimed GnRH-treated seasonally anestrous ewes, and that this problem might overcome by progesterone-induced changes in the vascularity of the preovulatory follicle.

Around the time of ovulation, extensive changes take place in the theca and granulosa layers of the preovulatory follicle to form the CL [17]. To meet the demands of these cells, a well-established vascular supply is essential for normal luteal development [25]. A disruption in the normal delicate balance of angiogenesis within the preovulatory follicle could lead to inadequate development of the follicular microvasculature, resulting in poor vascularization of the newly formed CL and thus be responsible for the observed defective luteal function [9], a deficiency that is overcome by progesterone priming. The current study was undertaken to test the hypothesis that progesterone priming of the GnRH-treated seasonally anestrous ewes alters the expression of angiogenic growth factors in the ovarian follicles. This was done by monitoring VEGF, the ANGs, and their receptors in small and large follicles of progesterone primed and nonprimed seasonally anestrous ewes treated with small-dose multiple injections of GnRH sufficient to induce ovulation.

2. Materials and methods

2.1. Animals and treatments

The experiment was conducted in August during the non-breeding season at Aberystwyth University, UK (latitude 52 °25′N) using 20 adult seasonally anestrous Welsh Mule ewes (Ovis aries). These ewes were in anoestrus at this time of the year because their breeding season starts in mid-October in the northern hemisphere. All treatments were given in compliance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research under the UK Home Office authorization (Animal Scientific Procedures Act, 1986). Half of the ewes received a single im injection of 20 mg progesterone (Sigma-Aldrich, Dorset, England) in corn oil 3 days before the start of GnRH treatment, whereas the other half received corn oil vehicle alone. Each ewe had its jugular vein cannulated and received 500 ng GnRH (LH-releasing hormone human acetate salt, Sigma-Aldrich) in 2 mL of sterile normal saline solution 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 to synchronize the onset of the preovulatory LH surge, as described previously [26]. Animals were killed with an overdose of pentobarbitral sodium (Merial Animal Health Ltd., Harlow, UK) and ovaries were collected from all the animals in each treatment group at either 24 or 46 hours after the start of GnRH treatment. These time periods were designed to provide follicles either 6 hours before or 16 hours after the onset of the preovulatory LH surge but before the time of ovulation, respectively.

2.2. LH monitoring

Blood samples were collected from all animals at 15-minute intervals for the first 4 hours from the start of GnRH treatment to monitor LH pulses (maximum concentration <3 ng/mL), and thereafter at 2-hour intervals until either euthanasia or 16 hours after the GnRH bolus injection to monitor the preovulatory LH surge (>10 ng/mL). Plasma was separated by centrifugation of the blood samples at 1500 rpm for 10 minutes at 4 °C and stored at −20 °C until analyzed for LH. We measured LH using a sandwich-type ELISA assay (LH DETECT, INRA Centre de Tours–PRC, Nouzilly, France) and performed as recommended by the manufacturer.

2.3. Sample collection

Immediately after collection, ovaries were washed in PBS pH 7.4 and fixed in 40% v/v neutral buffered formalin (BDH, Poole, Dorset, UK) for 24 hours and then preserved in 70% v/v ethanol until embedded in Paraffin wax (Paraplast, Leica, Taab Laboratories Equipment Ltd., Aldermaston, UK).

2.4. Slide preparation and ovary mapping

Wax-embedded tissue samples were sectioned (6 μm) and mounted onto SuperFrost Plus slides (BDH, VWR International, Lutterworth, UK) for immunohistochemistry and in situ hybridization analyses. To identify and
determine the size of follicles, ovaries were mapped by staining every 25th slide with hematoxylin and eosin, as previously described [27]. In summary, the hematoxylin and eosin-stained sections were placed under a stereoscope (Leica, Wetzlar, Germany), the follicles were identified and their diameter measured using an ocular scale. Follicles were tracked across sections and maximum follicular diameter was used to categories follicles into small (2–2.5 mm) and large (> 2.5 mm). Three cellular layers (granulosa, theca interna, and theca externa) of the follicles were used for analysis. A total of 91 small and 79 large follicles were used for immunohistochemistry and in situ hybridization analysis of each angiogenic factor.

2.5. Immunohistochemical analysis

Immunohistochemical assays for the proteins of interest were performed on consecutive sections of a follicle using the avidin-biotin peroxidase method as previously described [27–30], but with secondary antibody concentration of 1:25 biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in 2% v/v normal goat serum in PBS and color detection being performed using ImmPACT DAB (Vector Laboratories).

For VEGF, sections were incubated in 1:100 rabbit polyclonal anti-human VEGF antibody at 200 μg/mL (VEGFA-20, Santa Cruz Biotechnologies, Heidelberg, Germany) in PBS overnight at 4 °C and then left to develop color for 1 minute. For VEGF-R2, incubation took place overnight in 1:50 rabbit polyclonal anti-mouse FLK-1 (VEGFR-2) at 200 μg/mL (Santa Cruz Biotechnologies) at 4 °C. Slides were left to develop color for 2 minutes. For ANG-1, a 1:200 concentration of rabbit polyclonal anti-mouse ANG-1 at 85 mg/mL (Abcam Inc., Cambridge, MA) was used for 1 hour and slides were left to develop color for 2 minutes. For ANG-2, 1:100 Rabbit polyclonal anti-human ANG-2 at 200 μg/mL (Abcam) was used for 1 hour and color developed for 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 for 30 seconds to develop color. For the negative control sections the same protocol was followed except that the primary antibody was replaced by rabbit IgG (Santa Cruz Biotechnologies) at the same concentration. Positive staining was observed as brown color (Fig. 1B, D, F, J, L), whereas no staining was observed for negative controls (Fig. 1A, C, E, I, K).

2.6. In situ hybridization

In situ hybridization was performed only for ANG-1, ANG-2, and TIE-2. Bacteria containing ovine ANG-1, ANG-2, and TIE-2 complimentary DNA, as described previously [27], were provided by Dr. Chowdhury (Department of Veterinary Clinical Sciences, The Royal Veterinary College, North Mymms, Hertfordshire, UK). Riboprobes for ovine ANG-1, ANG-2, and TIE-2 were synthesized using T7 (sense) and SP6 (anti-sense) MEGAscript High-Yield Transcription Kits (Ambion Ltd., Cambridgeshire, UK) after linearization of plasmids with Sal I and Apa I, respectively. During synthesis, riboprobes were labeled with digoxigenin-11-uridine triphosphate (Roche Applied Bioscineces, Welwyn Garden City, England, UK) for nonradioactive detection following the manufacturer’s recommendations. In situ hybridization was performed according to the technique described by Kershaw, et al. [28] and Chowdhury, et al. [27]. ANG-1, ANG-2, and TIE-2 hybridization was performed for 19 hours in a humidiﬁed chamber at 60 °C and color development took place for 20 hours before mounting. Positive staining was observed as brown color for antisense riboprobes (Fig. 2B, D, F); no staining was observed for sense (control) probes (Fig. 2A, C, E).

2.7. Quantification of staining for immunohistochemistry and in situ hybridization

The pattern and intensity of protein and mRNA staining for VEGF, VEGFR-2, ANG-1, ANG-2, and TIE-2 in follicular tissue were determined semi-quantitatively using a histochecmal score (HSCORE) method that has been widely reported in a number of studies in our own laboratory [27–30] by others [31,32]. Moreover, the HSCORE method of analysis used in this study has been previously validated as a suitable method for semiquantitative estimation of immunohistochemistry and in situ hybridization results [33]. In this study, all assessments were carried out in a blinded manner by one experienced assessor using HSCORE, incorporating both the distribution and the intensity of specific staining as described previously [27,28,30,33,34]. Brieﬂy, the percentage of positively stained cells and the intensity of the staining of those cells were recorded at an original magniﬁcation of ×400. The percentage of speciﬁcally stained cells to which each intensity staining corresponded was also recorded. The intensity of staining was classiﬁed on a scale of 0 to 3, where 0 = no visible staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. Each cell layer from every follicle, granulosa, theca interna, and theca externa was analyzed at three randomly selected areas in each of three positive staining sections, resulting in a total of nine positive fields analyzed per cell layer for each follicle. An expression index was calculated for each follicle layer based on the percentage of positively stained cells and the intensity of staining using the following formula: Expression index = % total stained cells × (1% weak) + (2% medium) + (3% strong)) / 100. A mean expression index was calculated to represent the expression of each angiogenic factor and its mRNA in each follicular cell layer of every follicle from an individual animal.

2.8. Statistical Analysis

Data on both protein and mRNA for the different angiogenic factors were analyzed using a linear mixed model within the REML function of GenStat. Treatment × time × layer was used as fixed effect and animal/follicle number as random effects. Values were transformed to log (ANG-1 protein in small follicles and VEGF protein in large follicles) or square root (ANG-1 and -2 mRNA in small follicles and ANG-1 mRNA in large follicles) to normalize the data before statistical analyses. Overall effects of treatment (progesterone priming), time (before or after the LH surge),...
Fig. 1. Representative immunohistochemistry images of VEGF (A, B), VEGFR-2 (C, D), ANG-1 (E, F), ANG-2 (I, J), and TIE-2 (K, L) protein expression in ovarian follicles of progesterone primed seasonally anoestrous ewes treated with small-dose multiple injections of GnRH. Bright field microscopy at an original magnification of ×100 (C, D, E, F), ×200 (A and B), and ×400 (I, J, K, L). Positive and negative images are shown on the right and left, respectively. G, granulosa layer; T, theca layer.
and cell layer (granulosa, theca interna and theca externa) as well as three-way interactions (treatment × time × 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 the 5% level.

3. Results

3.1. General observations

As expected, small-dose multiple injections of GnRH successfully induced LH pulses, which did not exceed 3 ng/mL, whereas the GnRH bolus injection given at 30 hours after the start of GnRH treatment induced an LH surge (>10 ng/mL) in all the animals. Based on the LH profile for each group, it was clear that ovaries collected at 24 hours after the start of the GnRH treatment were collected before the LH surge and ovaries collected 46 hours after the start of GnRH treatment were collected 16 hours after the start of the LH surge and approximately 14 hours before the expected ovulation time. The lack of ovulation in these groups was further confirmed by the absence of a corpus haemorrhagicum and/or an ovulation point at the time of ovary collection.

3.2. Expression of angiogenic factors and their receptors in small follicles

In small follicles, VEGF protein expression was not affected by progesterone priming treatment, however, its expression was significantly greater (P < 0.05) in follicles collected before the LH surge. Neither the progesterone treatment nor the time had any effect on the protein or the mRNA expression of VEGFR-2, ANG-1, ANG-2, and TIE-2 in small follicles. Protein expression for VEGF, VEGFR-2, ANG-1, ANG-2, and TIE-2 was significantly greater (P < 0.05) in the theca interna than the granulosa or theca externa.
layers. Although mRNA expression for ANG-1 and ANG-2 was highest in the granulosa layer, there was no difference in the TIE-2 mRNA expression among the different follicular layers of the small follicles.

3.3. Expression of angiogenic factors and their receptors in large follicles

Expression of VEGF protein was significantly greater ($P < 0.05$) in large follicles of progesterone primed than nonprimed animals (Fig. 3), and in follicles collected before than after the LH surge (Fig. 4). In addition, VEGF protein expression was significantly ($P < 0.05$) greater in the theca interna compared with the theca externa and granulosa layers (data not shown), with no interaction ($P = 0.98$) between progesterone priming, time relative to the LH surge, and the follicular layer.

Although neither progesterone priming ($P = 0.14$) nor time of collection of follicles relative to the LH surge ($P = 0.55$) had any effect on the overall VEGF protein expression in large follicles, the theca interna had significantly ($P < 0.05$) higher protein expression than the other two follicular layers, with a significant ($P < 0.05$) three-way interaction between factors largely attributable to lower levels in the theca interna in follicles collected before the LH surge and higher levels in this same layer after the LH surge (Fig. 5).

Although there was no overall effect of either progesterone priming or time of collection of follicles relative to the LH surge on ANG-1 protein expression levels in large follicles, levels were significantly ($P < 0.05$) higher in the theca interna layer than either the theca externa or granulosa layers. In addition, there was a significant ($P < 0.05$) three-way interaction, and this was owing to the fact that ANG-1 levels were higher in the theca interna of follicles from progesterone primed ewes after the LH surge (Fig. 6).

Neither progesterone priming ($P = 0.17$) nor time of collection of follicles ($P = 0.40$) had any effect on the overall ANG-1 mRNA expression levels in large follicles, although levels of expression were significantly ($P < 0.05$) higher overall in the granulosa layer than either of the two thecal layers and in the theca interna compared with the theca externa layer. There was also a significant ($P < 0.05$) three-way interaction, again attributable to higher expression levels of ANG-1 mRNA in the theca interna of follicles from progesterone primed ewes after the LH peak (Fig. 7).

Although there was no effect of either progesterone priming ($P = 0.42$) or time of collection of follicles ($P = 0.89$) on the overall ANG-2 protein expression levels in large follicles, levels were significantly ($P < 0.05$) higher in the theca interna compared with either of the other two follicular layers. Again, there was a significant ($P < 0.05$) three-way interaction attributable to higher levels in the theca interna of progesterone primed ewes after the LH surge (Fig. 8).

The ANG-2 mRNA levels in large follicles were unaffected by either progesterone priming ($P = 0.40$) or time of collection.
collection (P = 0.12), although levels of expression were significantly (P < 0.05) higher in the granulosa compared with either of the two thecal layers but with no significant interactions between factors (data not shown).

Neither TIE-2 protein levels nor TIE-2 mRNA expression in large follicles were significantly affected by progesterone priming or timing of follicle collection relative to the LH peak. However, TIE-2 protein levels were significantly higher (P < 0.05) in the theca externa layer, whereas TIE-2 mRNA expression was significantly (P < 0.05) higher in the granulosa layer.

4. Discussion

The lack of extensive understanding of the factors involved in follicular maturation and their impact on subsequent luteal development [35] poses a limitation for fertility protocols in a number of species [1,36,37]. Seasonally anestrous ewes with and without progesterone priming before the induction of ovulation using either small-dose multiple injections of GnRH [7,9,10] or ram introduction [12] have been widely used as a model to study luteal dysfunction. We have recently reported that progesterone priming of seasonally anestrous ewes induced to ovulate with small-dose multiple injections of GnRH results in a greater vascular template being available for the newly developing CL [33]. However, the fact that progesterone given to seasonally anestrous ewes as early as 3 to 5 days before the start of GnRH treatment or ram introduction can successfully induce the formation of a fully functional CL [12,38] suggests that progesterone priming might ensure normal luteal function by primarily affecting events within the preovulatory follicles which will develop into these newly formed functional CL.

In this study, the 300 μg GnRH bolus injection given intravenously 30 hours after the start of the pulsatile GnRH treatment successfully resulted in synchronization of the onset of the LH surge in all animals at around the time that it would naturally occur, as reported previously [26]. This synchronization was of extreme importance to ensure a fixed time both before and after the LH surge that initiates the cascade of events leading to ovulation and structural reorganization of the follicle into the developing CL thereafter.

Adequate ovarian vasculature is essential for the delivery of hormones and nutrients to the developing follicle [15,22]. Although follicular blood vessels are limited to the
theca layers [17], the granulosa cells seem to be responsible for a significant production of angiogenic factors [15], which can diffuse to the theca compartment and/or accumulate in the follicular fluid creating an angiogenic gradient attracting blood vessels closer to the granulosa layer [39].

Angiogenesis is the formation of new blood vessels from an already existing vascular network [16,17,40], and is mainly regulated by the VEGF-ANG system [41]; VEGF is essential for follicular development [13,41] and the persistence of the theca interna capillary network is directly dependent on VEGF accumulation in the follicular fluid [39]. Although the granulosa layer is said to be the main source of VEGF protein production in the follicle [13,25], it was observed in this study that VEGF protein levels were significantly higher in the theca interna than in the other cellular layers of both small and large follicles. Because the theca interna is the main source of vascular supply to the developing follicle [39], higher expression of VEGF protein in the theca interna is not surprising but quite expected as a prerequisite for follicular angiogenesis. The comparatively lower VEGF protein levels observed in the granulosa layer in the current study could be the results of VEGF diffusion from its site of production to the theca interna and/or possible accumulation in the follicular fluid [39].

In small follicles, progesterone priming did not have any significant effect on VEGF expression before or after the LH surge. Although it has been reported that LH stimulates VEGF expression in the granulosa cells in vitro [15], VEGF expression in small follicles was found to be significantly higher before the LH surge. The LH receptors are known to appear in the granulosa cells of ovine follicles when they reach approximately 3.5 mm in diameter [13]; however, because 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 may not have had any direct effect of upregulating VEGF levels in small follicles, probably owing to the lack of LH receptors to facilitate such an effect. Furthermore, after the LH surge, small follicles either have their development halted or undergo atresia [10,13,42]. In either case, VEGF levels are expected to decline [43], as observed in this study.

We found VEGFR-2 to be expressed mainly by the theca layers of both small and large follicles, as previously reported [13,43] with the highest expression being found in the theca interna. Neither progesterone nor the LH surge had any significant effect on VEGFR-2 expression in small follicles.

In small and large follicles, the theca interna layer expressed significantly higher levels of ANG-1 and ANG-2 protein; however, TIE-2 expression did not significantly vary between the cell layers. ANG-1, ANG-2, and TIE-2 mRNA was mainly expressed by the granulosa layer, in agreement to previous reports on the ANGs mRNA expression in the follicular layers [22,23]. 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 [39] or to the follicular fluid. Neither progesterone priming nor the LH surge had any significant effect on ANG-1, ANG-2, or TIE-2 protein or mRNA expression levels in small follicles.

The great majority of large follicles in this study were 3.5 mm in diameter or greater and, therefore, expected to express LH receptors in the granulosa layer and be more responsive to LH [13], being potential preovulatory dominant follicles.

Large follicles of nonprimed animals showed significantly lower overall VEGF protein levels than those of progesterone primed animals. The observed lower VEGF levels in the large/potential preovulatory follicles of nonprimed animals may lead on one hand to the degradation of the existing vascular bed but more importantly to the poor vascular sprouting around the time of ovulation. Low VEGF levels during the late follicular phase have recently been shown to interfere with dominant follicle ovulation and have been associated with the development of nonfunctional CL [44].

Both small and large follicles had significantly higher VEGF expression before the LH surge. This is in agreement with previously reported data in which VEGF levels decrease around the periovulatory time within cells [44] but increase markedly in follicular fluid [15,40]. Although progesterone priming resulted in a significant increase in VEGFR-2 protein levels of large follicles after the LH surge, nonprimed animals showed a significant decrease in receptor levels. Furthermore, VEGFR-2 levels observed in the theca interna of large follicles of progesterone primed animals after the LH surge were significantly higher than those observed in nonprimed animals. LH is known to stimulate VEGF expression [15]. This study has shown that LH surge has a similar effect on VEGFR-2 production. Moreover, progesterone priming had an additive effect on the expression of both VEGF and VEGFR2, and these changes collectively are likely to exert their effects on biological function.

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 that becomes the base for luteal vascular development [22]. Significantly lower levels of VEGF and VEGFR-2 protein observed in nonprimed animals could significantly interfere with blood vessel sprouting, thereby limiting early luteal angiogenesis whereas an increase in VEGFR-2 protein production by the theca interna cells of progesterone primed animals may result in an overall increased effect of VEGF leading to a higher degree of vascularization and therefore, a normal luteal function in these animals.

Although in small follicles both progesterone priming and the LH surge do not seem to affect ANG-1 protein and ANG-1 mRNA expression levels, in large follicles progesterone priming significantly upregulated ANG-1 protein expression by the theca interna cells after the LH surge, possibly contributing to the early luteinization process that takes place in the cells of the ovulatory follicle [23]. The ANG-2 protein levels in progesterone primed animals after the LH surge were also significantly higher than those found in nonprimed animals. However, neither progesterone nor the LH surge had any significant effect on ANG-2 mRNA or TIE-2 protein and TIE-2 mRNA.
Growth and development of the CL depend on the establishment of an adequate vascular supply. Most blood vessels of the early CL are derived from the theca layer [45] and further vascular development originates from sprouting from this existing vascular network [16,17,40]. High VEGF and high Ang-2 protein levels are considered to be essential for neovascularization to occur [23]. The significantly lower levels of VEGF observed in the large follicles of nonprimed 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. At a time when angiogenesis is expected to be high and theca blood vessels are invading and sprouting into the granulosa layer [40], low levels of angiogenic factors after the LH surge could lead to poor sprouting of the theca capillaries and deficient neovascularization of the CL that forms from these preovulatory follicles. Furthermore, after the LH surge, ANG-1 and ANG-2 are thought to stimulate early luteinization and progesterone release from the periovulatory follicle [23]. Significantly lower levels of ANG-1 observed in nonprimed animals after the LH surge could further interfere or delay the initial luteinization process taking place in the follicular layers.

In conclusion, the mechanism by which progesterone priming eliminates defective luteal function has to date remained elusive. However, the results of this study indicate that the absence of progesterone priming leads to a defective production of angiogenic factors in large preovulatory follicles, especially in response to the LH surge, potentially compromising early luteal vascular development and therefore subsequent luteal function. Such effects are not likely to be limited to the model being used here because there is ample evidence that the first ovulation during periods of transition between anoestrus (occasioned by puberty, postpartum, or seasonality) and cyclic activity [3–5,12] are associated with an high incidence of defective luteal function. The results of the present study strongly support the concept that the low levels of progesterone secreted by this first short-cycle CL are the result of defective angiogenesis within the subsequent preovulatory follicle(s) that could be alleviated by progesterone exposure of these follicles before the LH surge.

Acknowledgments

The authors thank Dr. Walid Chowdhurry, Department of Veterinary Clinical Sciences, The Royal Veterinary College, for kindly providing bacteria containing the vector of interest for the Angiopoietins. A special thank you is also due to Mrs Hester Taylor and Mr Rafiul Alam for their help with sample analysis as well as for Mrs Tanya Hopcroft for assistance with the preparation of slides. This work was supported by a grant from the Wellcome Trust (GR06304MA). A.C.M. Christensen was supported by a studentship from Aberystwyth University, UK.

References

[18] Benjamini L, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the performed endothelial network and is regulated by Pdgf-B and VEGF. Developmen 1998;125:1591–8.


[29] Leethnongdee S, Kershaw-Young CM, Scaramuzzi RJ, Khalid M. Intra-cervical application of Misoprostol at estrus alters the content of cervical hyaluronan and the mRNA expression of follicle stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR) and cyclooxygenase-2 in the ewe. Theriogenology 2010;73:1257–66.


[34] Perry K, Haresign W, Waters DC, Khalid M. Hyaluronan (HA) content, the ratio of HA fragments and the expression of CD44 in the ovine cervix vary with stage of the oestrus cycle. Reproduction 2010;140:133–41.


