Follicular waves and concentrations of steroids and inhibin A in ovarian venous blood during the luteal phase of the oestrous cycle in ewes with an ovarian autotransplant

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Abstract

The dynamics of ovarian follicular development and the pattern of pituitary and ovarian hormone concentration were investigated during the luteal phase in ewes with autotransplanted ovaries. The follicles were measured by ultrasound and samples of ovarian and jugular venous blood were collected at intervals of 12 h. Blood samples were collected before and after a GnRH challenge (250 ng GnRH, i.v.) to allow the determination of basal and LH-stimulated concentration of ovarian steroids. Throughout the luteal phase, large antral follicles developed in three waves, each of which was preceded by a rise in the concentration of FSH ($P<0{\cdot}05$). The concentrations of oestradiol and androstenedione in the unstimulated and LH-stimulated samples were similar ($P>0{\cdot}05$) during the first 3 days of the luteal phase but differed thereafter, with the LH-stimulated being significantly higher than the basal concentrations ($P<0{\cdot}05$). In the first wave of follicular development the changes in follicular size were accompanied by an increase in the concentration of ovarian steroids and inhibin A. During the second follicular wave, although changes in follicle diameter were similar to the first wave ($P>0{\cdot}05$), the basal concentration of ovarian steroids and inhibin A remained unchanged throughout the period of emergence and demise of the large follicles. These results confirm that the development of large antral follicles during the luteal phase of the sheep occurs in successive waves that are associated with fluctuations in FSH secretion. However, while the results strongly suggest that fluctuations in both inhibin A and oestradiol secretion control FSH during the first follicular wave, the cause of the FSH fluctuations associated with waves two and three is unclear. Final resolution of this issue may need to await the development of a specific assay for dimeric inhibin B.

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Introduction

The luteal phase is characterised by an ovary bearing at least one corpus luteum (CL), which has evolved from the ovulatory follicle. The dynamics of the endocrine changes that occur during this phase of the cycle are well documented in sheep (for review see Goodman 1994). The secretion of progesterone increases progressively from ovulation to mid-cycle and reduces the frequency of luteinising hormone (LH) pulses to values incompatible with ovulation (McNeilly *et al.* 1991). Follicle-stimulating hormone (FSH) secretion is not affected by progesterone but is regulated by the oestradiol and inhibin produced by the follicles that develop during this period of the cycle (Baird *et al.* 1991).

The role of inhibin in regulating the production and secretion of FSH has been extensively documented in sheep, both *in vivo* (Martin *et al.* 1988, Mann *et al.* 1992, Tilbrook *et al.* 1993) and *in vitro* (Clarke *et al.* 1993, Muttukrishma & Knight 1994). The patterns of secretion of inhibin into the circulation, however, is less clear, due to limitations in the assays employed for its measurement. Most of the radioimmunoassays available are based on antibodies against purified or synthetic fragments of the α subunit and measure ‘so-called’ immunoreactive inhibin. They cross-react differentially to related proteins but all of them have a strong cross-reaction with non-bioactive forms of the α subunit. Although bioassays are able to measure the active forms of inhibin they can be influenced by the presence of activin, follistatin and ovarian steroids in the sample (Baird & Smith 1993).

On the basis of evidence from a number of techniques, it is thought that the development and demise of large antral follicles occurs several times throughout the luteal phase. Analysis of the population of ovarian follicles by histological methods demonstrated the occurrence of the development of large antral follicles during this period of the cycle, but failed to agree on its pattern or regulation (Smeaton & Robertson 1971, Brand & De Jong 1973, Turnbull *et al.* 1977). These follicles secrete oestradiol and

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androstenedione in response to LH stimulation (Baird & Scaramuzzi 1976, McNatty *et al.* 1981, Campbell *et al.* 1990*a*). The resulting secretion of oestradiol has been assumed to be responsible for the variation in FSH concentration with a period of 4–5 days throughout the luteal phase (Bister & Paquay 1983, Campbell *et al.* 1991*b*). Studies utilising transrectal ultrasonography have produced direct evidence of follicular growth during the luteal phase, with reports of random emergence of follicular growth (Schrick *et al.* 1993, Ravindra *et al.* 1994) or the emergence of between two and six waves of follicular development associated with fluctuations in FSH (Ginther *et al.* 1995). However, the relative importance of oestradiol and inhibin in the control of FSH concentration at different stages of the oestrous cycle is unknown.

Recently, studies utilising ewes with an ovarian autotransplant during the follicular and early luteal phase have shown that the development of waves of follicle growth is associated with increased secretion of oestradiol during periods of high LH pulse frequency (Souza *et al.* 1997). During anoestrus, when the frequency of endogenous LH pulses is low, the same effect was observed in samples collected after a gonadotrophin-releasing hormone (GnRH) challenge (Souza *et al.* 1996). Both these studies demonstrate a period of functional dominance, characterised by high oestradiol secretion, shorter than the period of morphological dominance when the follicle was still visible in the ovary. The aim of this study was to investigate the patterns of development of large antral follicles in relation to the concentrations of LH and FSH, ovarian steroids and dimeric inhibin A throughout the luteal phase.

Material and Methods

Experimental animals

Six mature Scottish Blackface Merino ewes (4–6 years old, ovulation rate ≈1·9) each with an ovarian autotransplant were studied during the breeding season (November). The animals were housed indoors at the Marshall Building, Roslin, Midlothian, Edinburgh, under natural lighting and received a maintenance diet consisting of hay and pelleted ration. The ovarian autotransplant (Goding *et al.* 1967) was performed at least 6 months prior to the study when the right ovary was removed and the left ovary relocated to a site under the skin of the neck. This preparation permits the collection of ovarian venous blood and facilitates determination of the ovarian follicle population by ultrasound. Because ewes with autotransplanted ovaries do not cycle spontaneously, due to separation of the uterus and ovaries (Baird *et al.* 1976), synchronisation of the oestrous cycle was achieved with two injections of cloprostenol, a potent analogue of prostaglandin $F_{2\alpha}$ (125 μg i.m. Estrumate, Cooper's Animal Health Ltd, Crewe, Cheshire, UK) given 17 days apart.

On the day prior to the start of blood sampling the left jugular vein was cannulated under local anaesthesia as previously described (Souza *et al.* 1996) and the ewes placed in metabolism crates. Three days after the second cloprostenol injection, at the expected time of ovulation, a second cannula was inserted into the contralateral jugular vein (transplant side) to enable collection of ovarian venous blood (Souza *et al.* 1996). The animals received a prophylactic treatment of broad spectrum long-acting antibiotic (3 ml i.m.; Clamoxil, SmithKline Beecham, Tadworth, Surrey, UK) every 3 days throughout the experiment.

Blood sampling

Samples of jugular (3 ml) venous blood were collected at 12-h intervals from the day prior to the second injection of cloprostenol until 18 days later. In addition, two sets of ovarian venous blood (5 ml) samples were collected every 12 h, starting 3 days after injection of cloprostenol. One sample of ovarian venous blood was collected under basal conditions (immediately after the jugular sample) and the other 30 min after a GnRH challenge (250 ng in 2 ml sterile saline i.v.; Sigma, Poole, Dorset, UK). In a preliminary trial, using animals of the same breed on day 15 of the luteal phase, this dose of GnRH produced LH pulses 15 min after the GnRH injection, with a mean amplitude of 4·4 ± 0·8 ng/ml (mean ± S.E.M., n=5) over a nadir of 0·7 ± 0·03 ng/ml. This dose of GnRH has been shown to induce an LH pulse of amplitude similar to that occurring spontaneously (McLeod *et al.* 1982) and was given to allow the measurement of basal and LH-stimulated levels of steroid hormone secretion. After sampling, each cannula was flushed with a 5 ml solution of 250 IU sodium heparin/ml in isotonic saline. The blood was centrifuged at 4 °C and the plasma separated and stored at − 20 °C until assayed.

Ultrasonography procedure

The skin over the transplanted ovary was clipped and shaved at the beginning of the experiment and maintained free of wool throughout. Before each ultrasound examination the area was covered with scanning gel (Siel Sound Gel; Siel Imaging Equipment Ltd, Aldermaston, Berks, UK). The exams were performed after each blood sample when the ovary was scanned in both horizontal (dorso/ventral) and vertical (cranio/caudal) planes, using a 7·5 MHz linear transducer (Model UST–5512 U–7·5; Aloka Inc., Tokyo, Japan) with a real time ultrasound scanner (SSD–500; Aloka Inc.). All ultrasound scans were recorded on video cassette tape for subsequent analysis.

The tapes were played in slow motion, the follicles were spatially located within the ovary and the image frozen at the largest section of the antral cavity for each individual follicle greater than 2·5 mm in diameter. The image was digitised into pixels of 256 shades of grey ranging from 0

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(white, maximum tissue density) to 255 (black, minimum tissue density) and using the NIH Image software (http://rsb.info.nih.gov/NIH-Image/download.html) the periphery of the follicle was identified. Measurements were taken for mean density of the pixels within the identified area and for the major and minor axes of the best fitted ellipsis for each follicle. The diameter of the follicles was determined as a mean of the two axes measured.

Immunoassay

Gonadotrophin and steroid plasma concentrations were measured in duplicate using previously described double-antibody RIA. FSH (Campbell *et al.* 1990*a*), LH (McNeilly & Fraser 1987) and progesterone were determined in unextracted jugular venous samples (Campbell *et al.* 1990*a*). Androstenedione (Campbell *et al.* 1990*a*) and oestradiol (Baird *et al.* 1981), were measured in ovarian venous plasma samples after solvent extraction. The sensitivities of the assays for FSH, LH, progesterone, androstenedione and oestradiol were 0·3 μg/l (USDA, oFSH, SIAFP–RP2), 0·2 μg/l (NIADDK, oLH, S23), 380 pmol/l, 175 pmol/l and 50 pmol/l respectively. The intra- and interassay coefficients of variation in the RIA were each less than 15% in the effective dose 20–80% range. Inhibin A was measured by enzyme-linked two-site immunoassay described for use in human plasma samples (Groome *et al.* 1996) and modified for use in sheep plasma (Knight *et al.* 1998). Briefly, the immunoassay is based on the use of an immobilised monoclonal antibody (E4) against the β-A subunit as a capture antibody, a biotinylated alpha C specific monoclonal antibody (17329/H2) as detection antibody and immunopurified 32 kDa bovine inhibin in ovariectomised sheep plasma as standard in the range 15·6–1000 pg/ml. In common with the human assay (Groome *et al.* 1996) and in contrast to the methodology described by Knight *et al.* 1998, the samples were denatured by boiling in 6% SDS for 3 min before oxidation with hydrogen peroxide. Standards and samples were denatured and oxidised before the incubation of the assay in a 96-well dish. The sensitivity of the assay was 30 pg/ml and the intra- and interassay coefficients of variation were 7·6 and 11·9% respectively.

Statistical analysis

The hormone data were normalised to the onset of the LH surge, defined as the nadir point before LH concentrations exceeded 10 ng/ml (day=0). For analysis of the relationship between diameter of the large follicles (that grew to a diameter of at least 5 mm) that developed during the luteal phase and hormone secretion, follicles were assigned to classes according to the day of the cycle each follicle emerged (when it was first observed with a diameter between 2·5 and 3 mm). The large follicles that emerged on days 1–5, 6–10 and 11–15 were assigned as follicle waves one (W1), two (W2) and three (W3) respectively. Within each group the data were analysed from the time of emergence of the large follicle until 5 days after. When more than one large follicle or CL per ewe was observed, data from all the structures were included to calculate the mean values, but the number of animals was used to calculate the standard error of the means. The effects of time on follicular diameter and hormone concentrations were analysed by repeated samples ANOVA on log transformed data using the general linear means model procedure of SYSTAT software (SYSTAT Inc., Evanston, IL, USA). Comparison of the number of follicles per ewe in each wave and the ovulation rate was performed by paired Student's *t*-test using the same software.

Results

Luteolysis was induced in all animals after the injection of cloprostenol (PG). However, one ewe did not have an LH surge and was excluded from the analysis. In the remaining five ewes the LH surge occurred 52·8 ± 2·9 h (mean ± S.E.M., n=5) after prostaglandin treatment. The diameter of the follicles which eventually ovulated increased from 4·4 ± 0·4 mm at the time of cloprostenol injection, to a mean diameter of 5·9 ± 0·3 mm at the onset of the LH surge and 5·8 ± 0·2 mm at the expected time of ovulation 24 h later (Fig. 1). The ovulation rate in the induced cycle was 2·4 ± 0·24 (mean ± S.E.M., n=5), with the CL progressively increasing in diameter between day 2 and 5, to around 12 mm, at which size they remained for the rest of the luteal phase (Fig. 1).

The mean concentration of progesterone in the peripheral blood remained low during the follicular phase and started to rise on day 4, progressively increasing until day 7 (P<0·05), when it stabilised around 20 nmol/l, remaining at this concentration for the rest of the luteal phase (Fig. 1). An increase in CL density, reflected by a decrease in pixel density, preceded the rise in progesterone production by one day, beginning at day 3, and continued to increase until day 5 (P<0·05) and remaining steady thereafter.

Ovarian hormones during the luteal phase

The concentrations of oestradiol and androstenedione (Fig. 2) were not affected by GnRH challenge in the interval between days 1 and 3·5 after LH surge (P>0·05), but thereafter the LH-stimulated steroid values were consistently higher than the unstimulated values (P<0·05). The oestradiol and androstenedione concentrations in basal samples increased progressively from day 1 until day 3·5 (P<0·05) and then decreased by day 7 (P<0·05) to low values similar to those observed at the onset of the luteal phase (Fig. 2). The mean concentration of oestradiol remained low for the rest of the luteal phase, apart from

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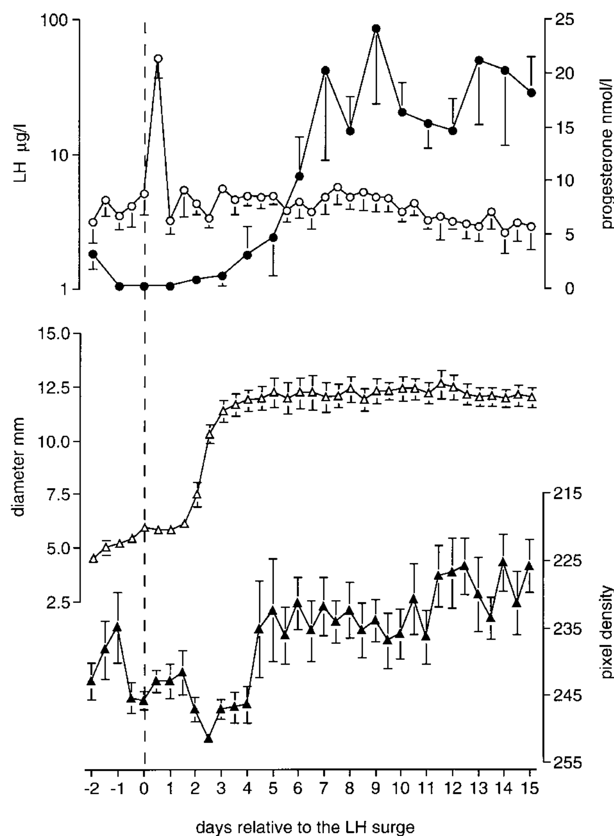

Figure 1 Relationship between concentration of progesterone (●) and LH (○) in jugular plasma and diameter (△) and pixel density (▲, 255=minimal tissue density) of the CL throughout the luteal phase. Note that the scale for pixel density has been inverted in order to reflect the increase in actual density of the CL during luteinisation. Values are means ± S.E.M., n=5.

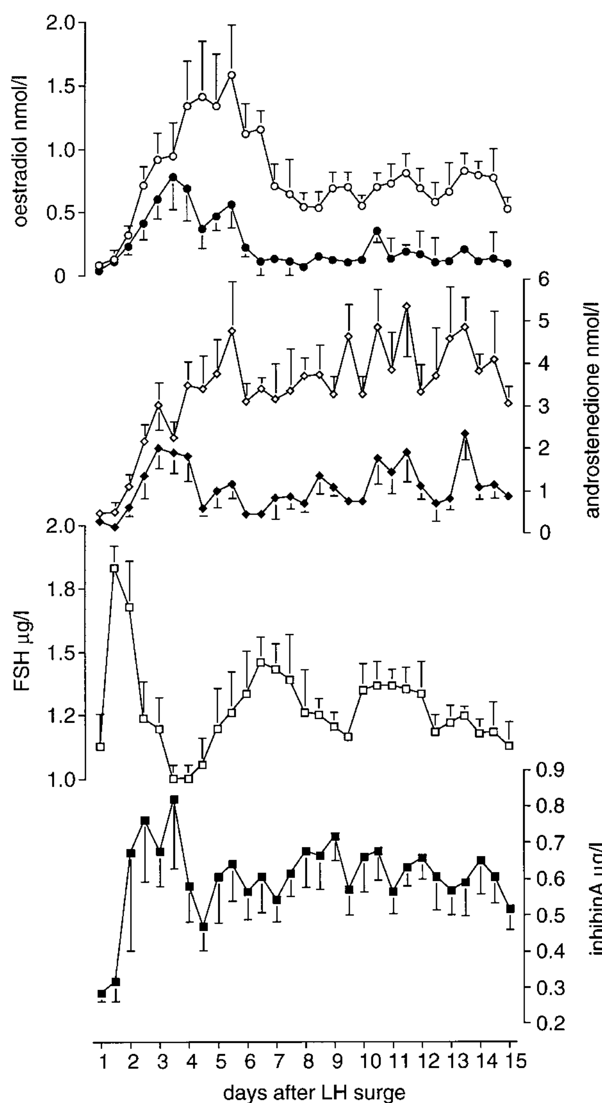

Figure 2 Concentration of FSH (□) in jugular venous plasma and concentration of oestradiol (○,●), androstenedione (◇,◆) and inhibin A (■) in ovarian venous plasma during the luteal phase (filled symbols showing basal steroid concentration and open symbol steroid concentrations following a GnRH-challenge, 250 ng i.v.). Values are means ± S.E.M., n=5.

some isolated points of high secretion, presumably where samples were collected after an endogenous LH pulse. Androstenedione concentration followed the same pattern as oestradiol until day 7, but the concentrations were higher than oestradiol during the rest of the luteal phase.

The concentrations of LH-stimulated oestradiol increased from day 1 until day 5 (P<0·05) followed by a progressive decrease until day 7 (P<0·05) and then remained at intermediate values for the rest of the luteal phase, never returning to the low values observed on day 1. Androstenedione concentration after GnRH challenge also increased progressively between days 1 and 5 (P<0·05) and remained at high and variable values throughout the luteal phase (Fig. 2).

The mean concentrations of inhibin A started at low values on day 1 (around 0·3 µg/l), but increased rapidly (P<0·05) to concentrations above 0·7 µg/l by day 2. Inhibin values remained high until day 4 when they declined to around 0·6 µg/l, remaining at this concentration for the rest of the luteal phase (Fig. 2).

Mean concentrations of FSH during the luteal phase were characterised by three distinct peaks (P<0·05). The first peak (secondary FSH surge), which occurred between days 1 and 2 of the cycle, was the most consistent and occurred at the time of minimal secretion of oestradiol and inhibin A. The second and third peaks occurred between days 5 and 8 and 10 and 12 respectively, but the timing in individual animals was variable and bore no clear relationship with mean basal or LH-stimulated steroid concentration (Fig. 2).

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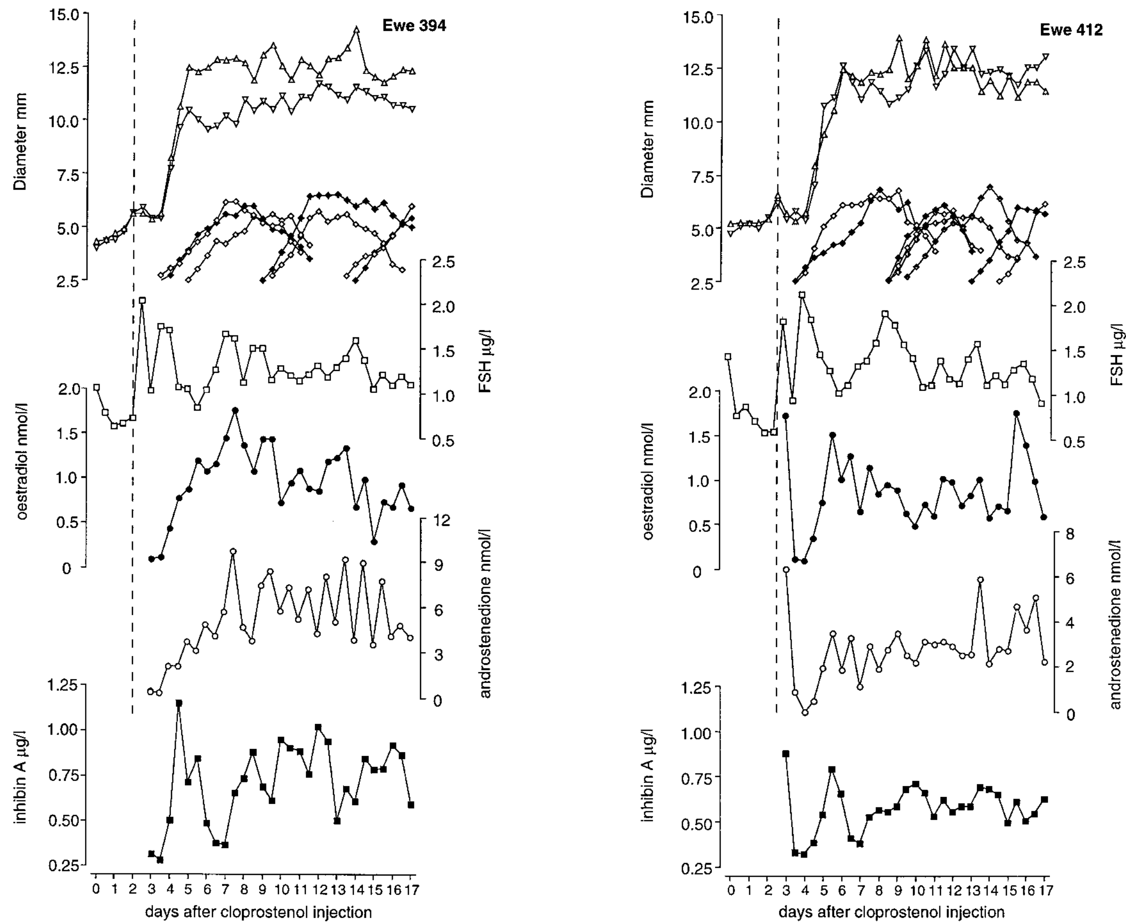

Figure 3 Dynamics of ovulatory follicles/CL (triangles) and dominant follicles (diamonds) from the three waves of follicular development during the luteal phase (top panel) and concentration of FSH in jugular venous plasma and concentration of oestradiol, androstenedione and inhibin A in ovarian venous plasma during the luteal phase from two representative animals (filled circles showing basal steroid concentration and open circles the steroid concentrations following a GnRH-challenge, 250 ng i.v.). The dotted line indicates the time of the onset of the LH surge.

Relationship between follicular development and hormonal secretion

The patterns of follicular growth and hormone secretion in two individual animals throughout the luteal phase are illustrated in Fig. 3. In all animals at least one large follicle emerged in each wave during the luteal phase, preceded by a rise in the concentration of FSH. The presence of follicles over 4 mm in diameter was synchronous with episodes of increased concentration of oestradiol after a GnRH challenge. For further analysis of the relationship between follicular waves and the hormone concentration, large follicles were divided according to the day of the cycle as presented in the analysis section and data were grouped around the time of emergence of each follicle.

In individual animals, follicle emergence for each of the three waves of follicular development was synchronised with large follicles from each wave emerging within 36 h of each other (Fig. 3). Due to variation in the wave interval, day of emergence was less synchronised among animals with differences over 48 h. Nevertheless there was a higher incidence of follicle emergence around days 2, 7 and 11 (Fig. 4). No follicles emerged on days 4, 5 and 9, days when the concentration of FSH was at its lowest.

A total of 29 large follicles were identified from five ewes during the luteal phase. Ten, eleven and eight follicles were allocated to the groups W1, W2 and W3 respectively. The mean number of follicles per ewe was $2{\cdot}0 \pm 0{\cdot}3$ in the group W1, $2{\cdot}2 \pm 0{\cdot}6$ follicles for group W2 and $1{\cdot}6 \pm 0{\cdot}2$ in the remaining group. The number of large follicles per ewe in each wave was similar ($P>0{\cdot}05$) and closely resembled the ovulation rate in the induced cycle ($P>0{\cdot}05$).

The large follicles in all three waves grew steadily for 2 days from emergence, at a rate of around 1 mm per day (Fig. 5). The FSH concentration was highest at the emergence of each wave and decreased ($P<0{\cdot}05$) as the

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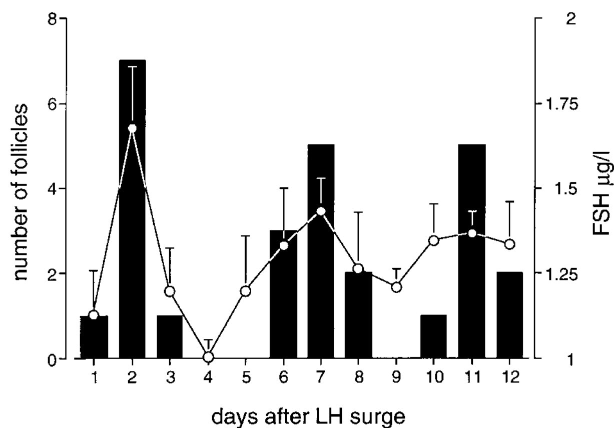

Figure 4 Relationship between emergence of dominant follicles (solid bars ≥ 5 mm in diameter) and mean concentration of FSH (○) in jugular venous blood (± S.E.M., n=5) during the luteal phase.

follicle grew to 5 mm in diameter. The relationship between the hormone concentrations and follicular development was detailed in the groups W1 and W2, where the full evolution of the follicular wave was observed (Fig. 5). The large follicles in both waves grew at a similar rate from emergence until 2 days later when they were around 5 mm in diameter. They remained over 5 mm in diameter for at least 2 days before decreasing in size, but the W2 follicles started to decrease in diameter 4·5 days after emergence (P<0·05) in contrast to the W1 follicles whose diameter remained unchanged at that time (Fig. 5). The ascending limb of the follicular wave was accompanied by a progressive increase in the concentration of unstimulated and LH-stimulated oestradiol and androstenedione in group W1 (P<0·05), while in group W2 the secretion of oestradiol remained constant throughout the development and demise of the large follicles (Fig. 5). The mean concentration of inhibin A increased during the first day of W1 (P<0·05) but did not change significantly during the remainder of both waves (Fig. 5).

The concentrations of FSH in both waves were high at the emergence of the large follicles and decreased as the follicles grew (P<0·05), remaining low while the follicles from W1 were actively secreting oestradiol. In W2 a similar reduction in FSH (P<0·05) was observed although in the absence of significant changes in either basal or LH-stimulated secretion of oestradiol (Fig. 5). However, the reduction in the concentration of FSH was greater (P<0·05) in W1 than in W2, between 2 and 4 days after emergence, when oestradiol was actively secreted by the large follicles.

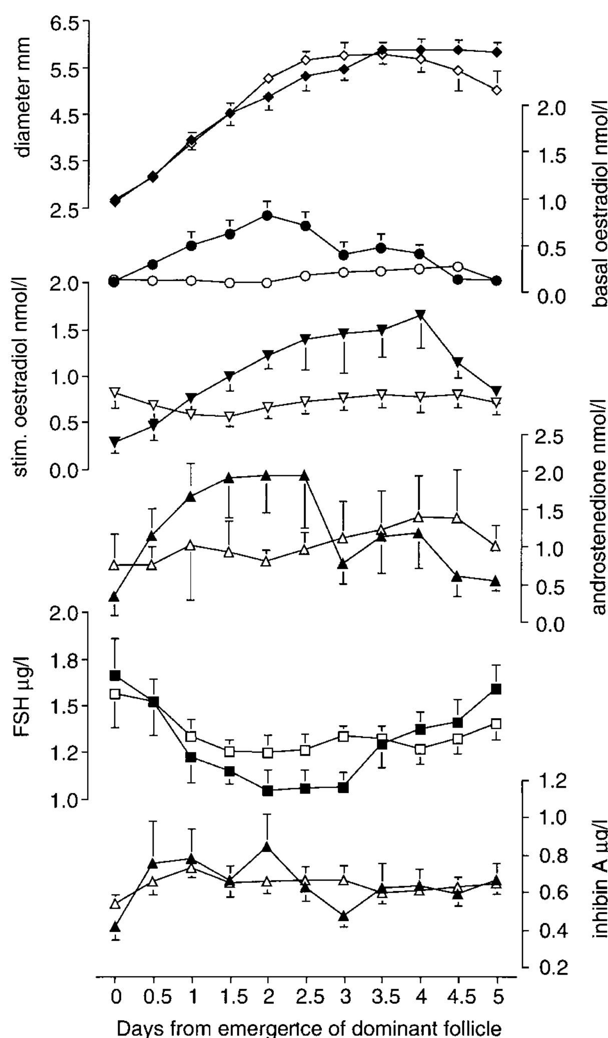

Figure 5 Relationship between diameter of the dominant follicle (◇), concentration of basal (○) and LH-stimulated (▽) oestradiol, basal androstenedione (△) and inhibin A (△) in ovarian venous plasma and concentration of FSH (□) in jugular venous plasma during the first wave (filled symbols) and second wave (open symbols) of follicular development of the luteal phase. Values are means ± S.E.M., n=5.

Discussion

This investigation demonstrates the existence of three follicular waves with similar rates of growth in the luteal phase of ewes with an ovarian autotransplant. Each of these waves is preceded by a rise in FSH concentration which decreases as the large follicle progressively increases in size. Although these data were obtained from an experimental model there is no evidence that the endocrine patterns of gonadotrophins or ovarian steroids are different between intact ewes and those with an ovarian autotransplant (Baird *et al.* 1976).

The observation of waves of follicular development in the sheep during the luteal phase is in agreement with previous reports using ultrasound to assess the follicular population (Ginther *et al.* 1995, Souza *et al.* 1997) and

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supports the hypothesis that in sheep, in contrast to primates, the concentrations of FSH during the luteal phase are high enough to allow development of large follicles (Baird *et al.* 1975). These results support the hypothesis proposed by Brown (1978) that a threshold concentration of FSH is required for development of dominant follicles, and that an increase in the concentrations of FSH of as little as 10% is able to trigger follicular development in hypogonadal subjects. Similarly, in hypogonadotrophic sheep the infusion of FSH equivalent to the peak levels during the mid–late luteal phase induces growth of preovulatory follicles (Picton & McNeilly 1991). However, the concentration of FSH necessary to keep large antral follicles growing is modulated by the LH pulse frequency (McNeilly *et al.* 1992, Campbell *et al.* 1995) and by intra-ovarian factors (Cahill *et al.* 1985, Findlay 1994, Campbell *et al.* 1995).

Although follicular waves during the ovine luteal phase have been reported earlier (Ginther *et al.* 1995) our data differ in a number of waves. This difference could be attributed to the great variability in cycle length reported in this previous study. The overall pattern of follicle turnover in sheep during the luteal phase seems to be similar to that observed in the cow which has two or three waves of follicle development (Sirois & Fortune 1988, Ginther *et al.* 1989). Nevertheless, there are clear differences between the species. In sheep, the wave interval is just 4–5 days, perhaps reflecting the smaller diameter of the dominant follicle, so that the ovulatory wave in sheep is likely to be the third or the fourth, not the second or third as in the cow. Furthermore, cows only have one dominant follicle per wave (Fortune 1993), whereas the sheep can have more than one dominant follicle per wave, depending on the ovulation rate of the breed.

It is well established that the secretion of FSH during the luteal phase is regulated by both oestradiol and inhibin (Baird *et al.* 1991, Mann *et al.* 1992) and fluctuations in the pattern of secretion of these two hormones would be expected to be responsible for the fluctuations in FSH that are associated with follicular waves. However, while the results of the present work strongly suggest that changes in both inhibin A and oestradiol secretion control FSH during the first follicular wave, the cause of the FSH fluctuations associated with waves two and three was less clear, a result which was not surprising given the differences in the origin and the trophic control of ovarian oestradiol and inhibin secretion. Ovarian oestradiol secretion is dependent on both the presence of a large oestrogenic follicle in the ovary and an appropriate level of LH stimulation (Baird & Scaramuzzi 1976). The relatively high secretion of unstimulated oestradiol and androstenedione during the first follicular wave (Cox *et al.* 1971, Campbell *et al.* 1990*a*, Schrick *et al.* 1993, Souza *et al.* 1997) is therefore almost certainly due to the fact that the frequency of LH pulses at this time is higher than that found later in the luteal phase, an idea supported by the fact that there was no difference between GnRH-stimulated and basal steroid secretion during the first 3–4 days of the follicular phase. Further, the fact that basal oestradiol secretion declined before stimulated oestradiol between days 4 and 6, strongly suggests that the decline in the concentration of oestradiol during the first wave is due initially to a lack of LH stimulation rather than atresia of the dominant follicle. Similarly, as LH pulse frequency remains low during the remainder of the luteal phase, the fact that basal secretion of oestradiol and androstenedione remained low thereafter was as expected. Surprisingly, however, while GnRH-stimulated androstenedione secretion remained high during the mid–late luteal phase, stimulated oestradiol secretion remained relatively low, suggesting that a period of exposure to a relatively high frequency of LH pulses is required before follicles acquire the ability to secrete oestradiol in normal amounts. This hypothesis is supported by the observation that large antral follicles obtained at mid and late luteal phase produce less oestradiol in short term culture compared with follicles from the early luteal phase (McNeilly *et al.* 1992). Thus, the lack of an association between ovarian oestradiol secretion and the development of large antral follicles during waves two and three can be explained on the basis of both a lack of LH drive and low follicular aromatase activity.

In contrast to oestradiol, we have previously demonstrated that, while oestrogenic large follicles are a major source, both small and large non-oestrogenic follicles contribute significantly to the ovarian secretion of immunoreactive inhibin (Campbell *et al.* 1991, Mann *et al.* 1992). Further, immunoreactive inhibin secretion is not acutely responsive to either FSH or LH *in vivo* (Campbell *et al.* 1989). While data on the source and control of ovarian dimeric inhibin A secretion in sheep are scarce, the available evidence suggests that dimeric inhibin A is also secreted by a range of antral follicles. First, expression of mRNA for both the inhibin α and β_A subunits has been observed in a range of antral follicles within an individual animal (Engelhardt *et al.* 1993, Tisdall *et al.* 1994). Secondly, granulosa cells from small antral follicles secrete significant quantities of inhibin A in short term culture, although FSH can stimulate inhibin A production as part of the normal differentiative cascade in long term culture (Campbell *et al.* 1997). Finally, the profile of inhibin A secretion obtained in the present experiment, low at day 1 and increasing with the emergence of the first wave but remaining high and constant during the rest of the cycle, suggests that ovarian inhibin A secretion only falls when atresia is induced in the antral follicle population by the preovulatory LH surge (McNatty *et al.* 1984), an interpretation supported by the sharp decrease in expression of mRNA for both α and β subunits of inhibin A in large antral follicles during and after the LH surge (Engelhardt *et al.* 1993). Thus, the lack of a clear association between inhibin A and the development of large

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antral follicles during waves two and three can be explained by the contribution of multiple follicles to ovarian inhibin A secretion. However, as inhibin A secretion remains high during the mid–late luteal phase when oestradiol secretion is negligible, it follows that inhibin A is the major regulator of FSH during waves two and three.

While it is possible that variations in ovarian inhibin B secretion could control FSH during the mid–late luteal phase, it appears unlikely, as inhibin B is ten times less potent than inhibin A in suppressing FSH release by cultured sheep pituitary cells (Robertson *et al.* 1996). Further, evidence from other species indicates that inhibin B is primarily produced by small antral follicles while inhibin A is the product of large oestrogenic follicles (human: Groome *et al.* 1996, rat: Woodruff *et al.* 1996). Resolution of the role of inhibin B in the control of FSH in sheep must await the development of specific assays. It is unlikely in our view, however, that subtle changes in the temporal relationships between FSH and oestradiol and inhibin secretion can be convincingly elucidated by an observational approach, as pooling of these data for analysis often destroys the relationships unless the key synchronising event can be identified. For instance, in the profiles of individual animals presented in Fig. 3, there do appear to be increases in inhibin A secretion that are associated with follicular waves two and three, but when the data are aligned by day of cycle (Fig. 2) or by day of emergence (Fig. 5), these associations are lost. Experimental approaches that perturb the normal feedback systems may, therefore, be more useful in answering these questions. Nevertheless, it is clear from the data presented that the number of large follicles in each wave during the luteal phase is similar to the ovulation rate, supporting the view that follicular selection occurs during the luteal phase (England *et al.* 1981). The key conundrum to be answered is, therefore, how the mechanism of follicle selection operates despite such distinct patterns of gonadotrophic and ovarian hormone secretion during the follicular phase and during the different waves of the luteal phase.

In ewes with an ovarian autotransplant the follicles that ovulate do not collapse after the LH surge (Souza *et al.* 1997). Nevertheless the secretion of progesterone throughout the luteal phase is similar to that in intact ewes (Collett *et al.* 1973, Baird *et al.* 1976, Campbell *et al.* 1990*a*). Moreover the presence of the cavity observed in the CL in this experiment is similar to that observed in intact ewes in which the majority of CL formed following ovulation after PG-induced luteolysis have cavities (Schrick *et al.* 1993). Further, despite the presence of luteal cavities there was no change in progesterone secretion and conception rates were not compromised (Schrick *et al.* 1993). The increase in the CL density prior to the increase in progesterone secretion is likely to be a reflection of the differentiation of the luteal cells leading to the colonisation of the luteal cavity (Niswender *et al.* 1994).

In conclusion, during the luteal phase there are three waves of follicular development, the emergence of which is preceded by a rise in the concentration of FSH. The concentrations of oestradiol and androstenedione in the unstimulated and LH-stimulated samples were similar during the first 3 days of the luteal phase but differed thereafter, with the LH-stimulated being significantly higher than the basal steroid concentrations, reflecting the endogenous low LH pulse frequency. In the first wave of follicular development the changes in follicular size were accompanied by an increase in ovarian steroid and inhibin A secretion. During the second follicle wave, although changes in follicle diameter were similar to those in the first wave, the secretion of ovarian steroids and inhibin A remained unchanged throughout the period of emergence and demise of the large follicles. These results confirm that the development of large antral follicles during the luteal phase of the sheep occurs in successive waves that are associated with fluctuations in FSH secretion. However, while the results strongly suggest that fluctuations in both inhibin A and oestradiol secretion control FSH during the first follicular wave, the cause of the FSH fluctuations associated with waves two and three is unclear. Final resolution of this issue may need to await the development of specific assay for dimeric inhibin B.

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