Follicular dynamics and ovarian steroid secretion in sheep during anoestrus

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The dynamics of ovarian follicular development and its relationship to ovarian and pituitary hormones during seasonal anoestrus were investigated for 10 days in nine ewes with autotransplanted ovaries in a longitudinal study. The size and position in the ovary of individual follicles over 2.5 mm in diameter were recorded by daily ultrasonography. Samples of ovarian and jugular venous blood were collected at intervals of 12 h, before and after a GnRH challenge (250 ng GnRH, i.v.) so that basal and LH-stimulated ovarian steroid secretion could be determined. Throughout the experimental period, all animals developed at least one large antral follicle > 5 mm, which secreted increased \( (P < 0.05) \) amounts of oestradiol and androstenedione in response to an LH challenge as the diameter of the follicle increased. However, a decrease \( (P < 0.05) \) in ovarian steroid secretion preceded any significant change in follicular diameter, indicating a dissociation between morphological and functional stages of dominance in sheep. We conclude that follicular growth and ovarian steroid secretion in sheep occur in wave-like forms, with the ascending and static part of both waves being synchronous but with a decline in steroid secretion preceding any changes in follicular diameter. Therefore, in sheep, follicular size alone is not an adequate parameter to assign dominance, and the secretory status of the follicle at any given time must be taken into account when studying the dynamics of follicular growth.

Introduction

The development of large antral follicles in sheep occurs throughout adult reproductive life but until recently the absence of suitable techniques has made the elucidation of the pattern of follicle development difficult. The advent of ultrasonography as a noninvasive and repetitive method of monitoring development of individual follicles has enabled a more comprehensive understanding of follicular dynamics in a number of species (Hackeloer et al., 1979; Griffin and Ginther, 1992). The use of this technique in cattle has led to the discovery of wave-like cycles of selection, dominance and regression of large antral follicles throughout the luteal phase (for review see Fortune, 1994). In sheep, however, the presence of follicular waves is still uncertain.

Although early evidence from histological (Brand and De Jong, 1973; Turnbull et al., 1977) and endocrinological (Cox et al., 1971; Mattner and Braden, 1972; Miller et al., 1981; Bister and Paquay, 1983) studies proposed that dominant follicle development in sheep did occur as a series of waves, more recent studies using transrectal ultrasonography have shown a random emergence of ovulatory-sized follicles (> 5 mm diameter) during the luteal phase (Schirck et al., 1993; Ravindra et al., 1994). Transrectal ultrasound studies in sheep are more difficult to perform and interpret than in cattle owing to problems of anatomical access and the smaller size difference between dominant and subordinate follicles. In addition, it is difficult to relate steroid production to the ovarian follicular population owing to the low concentrations of steroids in the peripheral blood in this species.

During periods of anoestrous in most breeds of sheep the pattern of follicle development is similar to that found in the breeding season, with no change in the total number of antral (McNatty et al., 1984a) or ovulatory-sized follicles (Smeaton and Robertson, 1971; Cahill, 1981; Noel et al., 1993). This follicular population is composed of both oestrogen active and inactive follicles, where the former are capable of producing oestradiol at the same rate as equivalent follicles in the breeding season (McNatty et al., 1984a). These oestrogen active or dominant follicles contain more LH receptors than do inactive ones (Webb et al., 1992), secrete oestradiol and androstenedione in acute response to LH pulses (Scaramuzzi and Baird, 1977) and can be induced to ovulate by pulsatile injection of LH (McNeilly et al., 1982; McNatty et al., 1984b) or GnRH (McLeod et al., 1982a) or by a bolus injection of hCG (Webb et al., 1992).

In the present study we used sheep in which the left ovary had been autotransplanted to a site under the skin in the neck (Goding et al., 1967) to examine the pattern of follicle development in anoestrous sheep. Unlike transrectal ultrasonography, this model has the advantages of (i) allowing easy access to an ovary which is fixed in position so that scans can
be performed in two planes and the spatial location of individual follicles can be determined, and (ii) allowing repeated collection of ovarian venous blood so that the secretory status of the ovarian follicle population can be determined. Anoestrous animals were chosen for this study to avoid perturbations in cycles of follicle development caused by the widespread atresia of nonovulatory follicles induced by the preovulatory LH surge (Baird and McNeilly, 1981).

**Materials and Methods**

**Experimental animals**

The experiment was performed during the nonbreeding season (June) at the Marshall Building, Roslin, Mid Lothian, Edinburgh. The anoestrous season in the Finn–Merino cross used in this study lasts from early April to late September. During anoestrum the ewes have, on average, an LH pulse of 6 µg l⁻¹ amplitude at 5 h intervals with the maximum oestradiol secretion in response to an LH pulse being observed after 25 min (Scaramuzza and Baird, 1977).

The animals were housed indoors, under natural lighting and received a maintenance diet consisting of hay and a pelleted ration. Nine ewes with ovarian autotransplants were studied for 10 days (Goding et al., 1987). The animals received cloprostenol, a potent PGF2α analogue, (125 µg i.m.; Estrumate, Coopers Animal Health Ltd, Crewe) 15 days before the start of the experiment and, to confirm that they were anoestrous, the concentration of progesterone in jugular venous plasma was determined in a sample collected 10 days after prostaglandin injection.

On the day before the start of blood sampling both ovarian and jugular veins were cannulated under local anaesthesia (2 ml s.c. of Lignocaine 2% Lignavet, Leyland) and the animals were placed in metabolism crates. The cannulae consisted of a 60 cm length Silastic tube (0.6 mm x 1.7 mm, internal and external diameter, respectively; 602-285, Sanitech, Hants). The ovarian venous cannula was introduced into the jugular vein of the loop anterior to the ovarian and jugular anastomosis and the tip was advanced until it was adjacent to the anastomosis, to allow collection of ovarian venous drainage. The jugular venous cannula was inserted into the contralateral jugular vein to a depth of 10 cm. After cannulation the animals received a bolus i.v. injection of 5000 IU of sodium heparin to prevent venous clot formation (Leo Laboratories Ltd, Bucks) and a broad spectrum long-acting antibiotic (3 ml i.m.; Clamoxil, SmithKline Beecham, Surrey). The prophylactic antibiotic treatment was repeated every 3 days throughout the experiment.

**Blood sampling**

Over the 10 day experimental period two sets of samples of both ovarian (5 ml) and jugular (3 ml) venous blood were collected at 12 h intervals, one under basal conditions and the other 30 min after a GnRH challenge (250 ng in 2 ml sterile saline i.v.; Sigma, Poole). This dose of GnRH has been shown to induce an LH pulse of 4–6 µg l⁻¹ in amplitude (McLeod et al., 1982b) and was given so that the unstimulated and LH-stimulated concentration of steroid hormones could be determined in ovarian venous plasma. After sampling, each cannula was flushed with 5 ml of a solution of 250 000 IU of sodium heparin l⁻¹ in isotonic saline, so the animals received 3000 IU of heparin every 12 h. The blood was centrifuged at 4°C for 15 min at 2000 g, the plasma separated and stored at −20°C until assayed.

**Scanning procedure**

The skin over the transplanted ovary was clipped and shaved at the beginning of the experiment, and the latter procedure repeated every 2 days during the course of the experiment. Before each examination the area was covered with scanning gel. The ovary was scanned daily in both horizontal and vertical planes, using a 7.5 MHz linear transducer (Model UST-5512U-7.5; Aloka Co. Ltd, Tokyo) with a real time ultrasound scanner (Aloka SSD-500; Aloka Co. Ltd). All examinations were recorded on video cassette tape and stored for subsequent analysis of the follicular diameter.

The tapes were played in slow motion and the image of follicles > 2.5 mm frozen at the largest section of the antral cavity for each individual follicle, which was located within the ovary and measured in the medio-lateral, dorso-ventral and cranio-caudal planes. The diameter of the follicles was determined as a mean of three measurements.

**Radioimmunoassay**

Gonadotrophin and steroid plasma concentrations were measured in duplicate using previously described double-antibody radioimmunoassays (RIA) for FSH (Campbell et al., 1990), LH (McNeilly and Fraser, 1987) and progesterone which were determined in unextracted jugular samples (Campbell et al., 1990). Androstenedione (Campbell et al., 1990), stimulated oestradiol (Baird et al., 1981), and unstimulated oestradiol (Beard and Lamming, 1994) were measured in ovarian venous plasma samples after solvent extraction by established RIA. Both oestradiol assays were performed using 100 µl of ovarian plasma extracted with 1 ml di-ethyl ether. The recovery rate after extraction was 92% ± 0.3 (mean ± SEM, n = 20) and hence the results were not corrected for extraction losses.

The sensitivities of the assays for FSH, LH, progesterone, androstenedione and LH-stimulated oestradiol and unstimulated oestradiol were 0.3 µg l⁻¹ (USDA, oFSH, SIAFP-RP2), 0.2 µg l⁻¹ (NIADDK, oLH, S23), 380 pmol l⁻¹, 175 pmol l⁻¹, 50 pmol l⁻¹ and 0.5 pmol l⁻¹, respectively. The intra- and interassay coefficients of variation were < 15% in the ED20–80 range.

**Statistical analysis**

Although cycles of development and regression of large antral follicles were evident in profiles from individual animals, these cycles were not synchronized in different animals. The data were grouped from all animals by identifying dominant follicles using three parameters: (1) achievement of a diameter of 5 mm; (2) maintenance of a diameter ≥5 mm for 2 days; (3) at least one measurement having been made before it achieved a diameter of 5 mm.
Using these parameters, designed to ensure that the population analysed was composed of growing healthy follicles that achieved dominance, 12 dominant follicles were identified, as some ewes had more than one wave during the observation period. The size of the largest follicle and the hormone concentrations were aligned according to the day the dominant follicle achieved a diameter of 5 mm (day 0) and the data incorporated from 1 day before until 3 days after day 0 (days -1–3).

The effect of time on the dominant and largest follicle diameter and the concentration of hormones were analysed by repeated samples ANOVA using the general linear means model procedure of SYSTAT software (SYSTAT Inc., Evanston, IL).

Results

All animals but one remained in anoestrus during the experimental period and the ewe that ovulated was withdrawn from the analysis.

Pattern of follicle development and hormone secretion

Over the 10 day observation period the ovaries of the experimental ewes contained on average 2.1 ± 0.39 follicles that remained between 3 mm and 5 mm (medium) and 4.25 ± 0.36 follicles that achieved diameter > 5 mm (large). In individual animals there were clear cycles of development and regression of large dominant follicles with a period of between 5 and 10 days (Fig. 1; Table 1).

Measurements were available for at least 4 days during the growth phase of only five follicles. The mean size of the dominant follicles on day -3 was 3.3 ± 0.2 mm (mean ± SEM, n = 5) and they grew in a linear fashion at a rate of 0.64 mm day⁻¹ until they achieved a diameter of 5 mm on day 0 (Fig. 2).

LH and progesterone remained at basal concentrations throughout the sampling period and did not vary with time (profiles not shown). The overall concentrations (mean ± SEM, n = 8) were 1.91 ± 0.27 μg LH 1⁻¹ and 0.52 ± 0.1 nmol progesterone 1⁻¹.

In addition to the follicular waves, there were also wave-like changes in the amount of GnRH-stimulated oestradiol and androstenedione secretion. In individual animals the follicular and secretory waves were positively related during the growth phase of a large antral follicle but unrelated thereafter. Jugular venous FSH concentrations remained relatively stable throughout the experimental period and there was no clear relationship with the pattern of follicular enlargement or steroid secretion (Fig. 1).

Relationship between development of dominant follicle and steroid secretion

In order to clarify the relationship between follicular waves and ovarian hormone secretion, dominant follicles were identified using the criteria presented in the analysis section and data were grouped around the time of emergence of each dominant follicle (Fig. 3). Neither the size of the largest follicle nor unstimulated oestradiol secretion changed (P > 0.05) during the period of emergence or regression of a dominant follicle. In contrast, as the dominant follicle grew between day -1 and day 0, the secretion of stimulated oestradiol increased to a peak on day 1 (P < 0.05) and then declined (P < 0.05) between day 1 and day 2, while the size of the dominant follicle remained constant, demonstrating a close association between stimulated oestradiol secretion and follicular growth during the ascending part of the wave. In addition to oestradiol secretion, stimulated androstenedione secretion was also positively related to follicular development and exhibited a similar profile to stimulated oestradiol with an increase during the growing phase of a dominant follicle and a decline preceding any decrease in follicular size. Unlike oestradiol, however, androstenedione secretion increased again at day 3 (P < 0.05).

Jugular venous FSH concentrations did not show a clear association with the pattern of follicular enlargement or stimulated oestradiol, but did show a small but significant
Table 1. Number and duration of dominant follicular waves per animal

<table>
<thead>
<tr>
<th>Ewe id.</th>
<th>Number of waves</th>
<th>Length in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2</td>
<td>6; 7</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>9; 6</td>
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<tr>
<td>9</td>
<td>1</td>
<td>5</td>
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<tr>
<td>11</td>
<td>1</td>
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<td>21</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>58</td>
<td>2</td>
<td>5; 7</td>
</tr>
<tr>
<td>Total (mean)</td>
<td>12</td>
<td>(7.1 ± 0.2)</td>
</tr>
</tbody>
</table>

The wave length was defined as the time that the dominant follicle remained over 3.5 mm in diameter.

change with time, decreasing between day – 1 and day – 0.5 and increasing from day – 0.5 to day 0 (P < 0.05).

Discussion

In this experiment, by using the ovarian autotransplant model, we have been able to demonstrate the existence of follicular waves in sheep and show that size and steroidogenic capacity of large antral follicles in sheep are only positively related during periods of follicle growth, thus defining stages of both functional and morphological dominance in sheep during anoestrus.

The observation that a follicle, identified as dominant on the basis of its size, may not be functionally dominant (steroidogenic) has also been made in cattle (Fortune, 1994). However, in that mono-ovulatory species, ultrasound alone can be reliably used to identify the stage of the follicular wave owing to the fact that the single dominant follicle is substantially larger than subordinate follicles and that the duration of the follicular wave is relatively long and well defined. In contrast, in sheep, the small size difference between dominant and subordinate follicles and the short and variable period of the waves makes identification of the dominant follicle by size alone difficult. It is therefore not surprising that studies which have attempted to characterize follicle dynamics in sheep solely on the basis of size have variably concluded that follicular growth is either continuous and independent of the stage of the oestrous cycle (Turnbull et al., 1977; Lahlou-Kassi and Mariana, 1984; Schirck et al., 1993) or consists of two (Brand and De Jong, 1973), three (Smeaton and Robertson, 1971; Noel et al., 1993) or 3–6 (Ginther et al., 1995) waves per cycle. Although we have used the term ‘dominant follicle’ in this paper to describe a large oestrogenic follicle it is clear that follicle development in sheep is a very dynamic process, and this could explain the reported lack of follicular dominance in sheep (Driancourt et al., 1991; Driancourt, 1994). However, it is important to emphasize that stimulated oestradiol secretion is probably a marker for functional dominance and not its cause, since dominance is likely to be exerted and modulated at a local level (Campbell et al., 1995).

![Fig. 2. Photographs of sequential daily scans (a–d) showing the enlargement of the second dominant follicle (days 4–7) presented in Figure 1. The large arrowhead indicates the dominant follicle while subordinate follicles are indicated by small arrowheads. S, skin; O, margin of the ovary. The grid marks visible at the top of each scan indicate a distance of 10 mm.](image)

The necessity for FSH to drive the growth of follicles > 2.5 mm (gonadotrophin dependent; Scaramuzzi et al., 1993) has been demonstrated in several models such as chronic hypophysectomy (Dufour et al., 1979), active immunization against GnRH (McNeilly et al., 1986) or chronic GnRH-agonist infusion (McNeilly and Fraser, 1987). In the present study we did not observe cyclic fluctuations in the concentration of FSH, as reported in nonprolific breeds (Bister and Paquay, 1983; Campbell et al., 1991a), nor any direct link with FSH secretion.
suggested by data generated from studies on the expression of steroidogenic enzymes in cows during the first follicular wave (Xu et al., 1995). The generally higher androstenedione secretion and the rise in androstenedione on day 3 when oestradiol remained at basal concentrations can be attributed to the fact that oestradiol is mainly secreted by the dominant follicle whereas ovarian androstenedione secretion receives a significant contribution from smaller follicles (Baird and Scaramuzzi, 1976; Baird et al., 1976; Campbell et al., 1991b). Thus the increase in the concentration of androstenedione on day 3 was probably due to evolving follicles from the next wave.

We conclude that follicular growth and ovarian steroid secretion in sheep occur in wave-like forms with the ascending and static part of both waves being synchronous but with a decline in steroid secretion preceding any changes in follicular diameter. Therefore, in sheep, follicular size alone is not a good parameter to assign dominance, and the secretory status of the follicle at any given time must be taken into account when studying the dynamics of follicular growth.

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References

Baird DT and Scaramuzzi RJ (1976) The source of ovarian oestradiol and androstenedione in the sheep during the luteal phase Acta Endocrinologica 83 402-406
Bister JL and Paquay R (1983) Fluctuations in the plasma levels of the follicle-stimulating hormone during estrous cycle, anestrus, gestation and lactation in the ewe: evidence for an endogenous rhythm of FSH release Theriogenology 19 565-582
Brand A and de Jong WHR (1973) Qualitative and quantitative micromorphological investigations of the tertiary follicle population during the oestrous cycle in sheep Journal of Reproduction and Fertility 33 431-439
Campbell BK, Mann GE, McNeilly AS and Baird DT (1990) The pattern of ovarian inhibin, estradiol, and androstenedione secretion during the estrous cycle of the ewe Endocrinology 127 227-235
Campbell BK, Scaramuzzi RJ, Evans G and Downing JA (1991a) Increased ovulation rate in androstenedione-immune ewes is not due to elevated plasma concentrations of FSH Journal of Reproduction and Fertility 91 653-666
Campbell BK, McNeilly AS, Mann GE and Baird DT (1991b) The effect of stage of estrous cycle and follicular maturation on ovarian inhibin production in sheep Biology of Reproduction 44 485-490

Cox RJ, Mattner PE and Thorburn GD (1971) Changes in ovarian secretion of oestradiol-17β around oestrus in the sheep Journal of Endocrinology 49 345–346


Ginther OJ, Kot K and Wiltham BC (1995) Associations between emergence of follicular waves and fluctuations in FSH concentrations during the estrous cycle in ewes Theriogenology 43 689–703


McNeilly AS, O’Connell M and Baird DT (1982) Induction of ovulation and normal luteal function by pulsed injections of luteinizing hormone in anoestrous ewes Endocrinology 110 1292–1299


Mattner PE and Braden AWH (1972) Secretion of oestradiol-17β by the ovary during the luteal phase of the oestrous cycle in relation to ovulation Journal of Reproduction and Fertility 28 136–137


Scaramuzzi RJ and Baird DT (1977) Pulsahtle release of luteinizing hormone and the secretion of ovarian steroids in sheep during anestrus Endocrinology 101 1801–1806


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