Incipient ovarian failure associated with raised levels of follicle stimulating hormone and reduced levels of inhibin A in older sheep

Carlos J.H.de Souza, Bruce K.Campbell and David T.Baird

Department of Obstetrics and Gynaecology, University of Edinburgh, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, UK

Introduction

The population of ovarian follicles in mammalian species is established before the onset of puberty and this limited reserve is continuously depleted by the commitment of primordial follicles during adult reproductive life (Hirshfield, 1991). However, humans are unique among other mammalian species and normally experience the extinction of the ovarian follicle population, resulting in a reproductive status known as menopause (Gosden and Faddy, 1994). Menopause generally does not occur as an abrupt change, but after a variable period of menopausal transition. This is initiated when changes in cycle frequency or menstrual flow are first observed, with both gonadotrophins, oestradiol and immunoreactive inhibin showing a marked degree of variability with shifts from typical post-menopausal patterns to those characteristic of reproductive age. Within 1–2 years after the final menstrual period or menopause, follicle stimulating hormone (FSH) concentrations are markedly elevated and luteinizing hormone (LH) levels moderately so, while oestradiol and immunoreactive inhibin values are low or undetectable, reflecting the exhaustion of the follicle reserve (for review see Burger, 1996).

The initial endocrine changes of the pituitary–ovarian axis in women first become apparent around the age of 38 years, with a selective rise in serum FSH concentrations occurring about the same time as a marked acceleration in the loss of primordial follicles from the ovary and increased atresia in the secondary follicles’ population (Sherman et al., 1976; Ebbyary et al., 1994; Gougeon et al., 1994; Faddy and Gosden, 1995). The monotrophic FSH rise in normally ovulating older women (age 40–45 years) is associated with decreased peripheral concentrations of inhibin B during the luteal phase, while oestradiol concentration is higher and inhibin A secretion is similar to a comparative younger cohort of subjects (age 20–25 years) (Klein et al., 1996). These findings suggest that decreased inhibin B secretion reflects a diminished follicular pool in older women and may be an important regulator of the monotrophic FSH rise. However, to date there is no reliable hormonal marker for the size of the primordial follicular pool to predict the reproductive life span, and the lack of animal experimental models to relate follicular population in the ovary and ovarian hormone secretion has meant that development in this area has been slow.

We have had available a group of hemiovariectomized ewes, in which the left ovary was autotransplanted to the neck at least 10 years previously and which have been maintained in good health until an age of 12–13 years and the left ovary was autotransplanted to the neck at least 10 years previously, which have been maintained in good health until an age of 12–13 years. Two experiments were conducted with these animals to determine the endocrine and follicular effects of age: a retrospective experiment in the same Finn-Merino ewes (n = 5) when the animals were 6–7 or 12–13 years of age; and a cohort experiment in old (12–13 years, n = 6) and young (2 years, n = 5) ewes of the same breed. In both retrospective and cohort experiments, the concentrations of FSH were significantly higher (P < 0.05) in older animals during the luteal phase when oestradiol secretion was low. This increase in FSH was associated with a decrease in the concentration of inhibin A (P < 0.05) in older animals in both the follicular and luteal stages of the cycle but the concentrations of oestradiol were similar between ages. Although there were significantly fewer antral follicles (P < 0.05) available for development in older ewes during the early luteal phase of the cycle, the ovulation rate was similar to that observed in younger animals (2.0 ± 0 vs 2.0 ± 4; P > 0.05) but the interval from luteal regression to the onset of the LH surge was longer (P < 0.05) in older animals. In conclusion, the endocrine changes associated with increasing reproductive age in sheep are therefore similar to those observed in women, suggesting that the sheep could be a useful animal model to study the effect of age on human fertility.

Keywords: FSH/inhibin A/menopause/oestradiol/sheep
Materials and methods

Experimental animals

Finn-Merino cross ewes with an ovarian autotransplant were studied during the breeding season. 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 left ovary had been autotransplanted by anastomosing the ovarian artery and utero-ovarian vein to the right carotid artery and jugular vein, respectively. The right ovary was removed at the time of autotransplantation and, hence, the total ovarian secretion of steroids could be measured by cannulating the right jugular vein into which the ovarian vein of the transplanted ovary drained (Goding et al., 1967). This preparation permits the collection of ovarian venous blood and facilitates determination of the ovarian follicle population by ultrasound. Because ewes with autotransplanted ovaries show persistence of the corpus luteum due to the separation of the uterus and ovaries (Baird et al., 1976), luteal regression and synchronization 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 ovarian vein and the left jugular contralateral to the transplanted ovary were cannulated under local anaesthesia as previously described (Campbell et al., 1995). The ewes were placed in metabolism crates and received a prophylactic treatment of broad-spectrum long-acting antibiotic (3 ml i.m.; Clamoxil, SmithKline Beecham, Welwyn Garden City, Surrey, UK) every 3 days throughout the experiment. Samples of jugular and ovarian venous blood were collected as previously described (de Souza et al., 1997a); the blood was centrifuged at 4°C, and the plasma was separated and stored at -20°C until assay.

Immunoaassay

Gonadotrophin and steroid plasma concentrations were measured in duplicate using previously described double-antibody radioimmunoassay. FSH (Campbell et al., 1990a), LH (McNeill and Fraser, 1987) and progesterone were determined in unextracted jugular venous samples (Campbell et al., 1990a). Androstenedione (Campbell et al., 1990a) 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 (NIDDK, oLH, S23), 380 pmol/l, 175 pmol/l and 50 pmol/l, respectively. The concentration of inhibin A in ovarian venous plasma was measured by two-site enzyme-linked immunosorbent assay (ELISA) described for use in human plasma samples (Groome et al., 1994) and modified for use in sheep plasma (Souza et al., 1997b). The sensitivity of the ELISA was 30 ng/l and the intra- and inter-assay variation of the immunoassays used were less than 15% in the effective displacement (ED) 20–80 range.

Experiment 1 (retrospective)

Samples of ovarian venous plasma were collected in November 1994 during the luteal (day 12) and follicular phases (24 and 48 h after cloprostenol injection) from ewes ($n = 5$) which were 12–13 years of age (de Souza et al., 1997a) and compared with samples collected in November 1989 at equivalent stages of the cycle from the same animals 5 years previously (Campbell et al., 1990b, 1991b). Both sets of samples were included in the same assay for FSH, oestradiol and inhibin A.

The effect of age on the hormone concentration at different stages of the oestrous cycle was analysed by paired $t$-test using the Systat software (Systat Inc., Evanston, IL, USA).

Experiment 2 (cohort)

During the breeding season (November 1996), five Finn-Merino cross ewes (2 years old) with an ovarian autotransplant (Goding et al., 1967) which had been performed at least 6 months previously were studied throughout the follicular and early luteal phases of the oestrous cycle. Samples of ovarian (7 ml) and jugular (3 ml) venous blood were collected at 6 hourly intervals for 10 days starting on the day before induction of luteal regression with the second cloprostenol injection (day 15). The blood was centrifuged at 4°C, and the plasma was separated and stored at -20°C until assay. The concentrations of FSH, LH and progesterone in jugular plasma and concentrations of oestradiol, androstenedione and inhibin A in ovarian venous plasma were measured by immunoassay. The pattern of hormone concentration and follicular dynamics of this cohort of young ewes was compared with those from old animals from the same breed (12–13 years, $n = 6$) at equivalent stages of the cycle (de Souza et al., 1997).

Ultrasound scanning

The details of the ultrasound measurement of follicle diameter have been described in previous publications (de Souza et al., 1997a). Briefly, the skin over the transplanted ovary was shaved and then the ovary scanned in both horizontal (dorso/ventral) and vertical (cranio/caudal) planes using a 7.5-MHz linear transducer (Model UST-5512U-7.5, Aloka Inc., Tokyo, Japan) with a real-time scanner (SSD-500; Aloka Inc.). All scans were recorded on videocassette for subsequent analysis, which involved identifying the location of each individual follicle 2.5-mm diameter. The diameter was taken as the mean of these measurements in medio-lateral, dorso-ventral and cranio-caudal planes. In this way, serial measurements of individual follicles could be recorded.

The data were normalized with respect to the time of cloprostenol injection and 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 early luteal phase and hormone secretion, follicles were aligned by emergence (first time a follicle was observed in the scans with a diameter between 2.5–3 mm). When more than one dominant follicle or corpus luteum (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 age of the cohort and time of the cycle on follicular diameter and hormone concentrations were analysed by repeated samples analysis of variance using the Systat software (Systat Inc.). Comparison of the interval luteolysis-onset LH surge and the ovulation rate between the age groups was performed by independent Student’s $t$-test using the same software.

Results

Experiment 1 (retrospective)

The concentrations of the hormones at both ages are presented in Table 1. The concentrations of FSH were significantly higher ($P < 0.05$) in the samples taken at an older age, with this increase being particularly marked ($P < 0.01$) during the luteal phase. The magnitude of the difference in FSH concentration decreased during the follicular phase but remained distinct between the ages ($P < 0.05$). Increasing age was also associated with a decrease in the concentration of inhibin A during the
Table I. Concentration of hormones (mean ± SEM) in the ovarian vein at different stages of the oestrous cycle in the same ewes (n = 5) when they were 6–7 and 12–13 years old

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Age (years)</th>
<th>Luteal</th>
<th>PG + 24 h</th>
<th>PG + 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibin A µg/l</td>
<td>6–7</td>
<td>826.6 ± 107.6</td>
<td>842 ± 202.6</td>
<td>953.2 ± 270.7</td>
</tr>
<tr>
<td></td>
<td>12–13</td>
<td>479.3 ± 157.6*</td>
<td>684.7 ± 204.8</td>
<td>899.3 ± 282.2</td>
</tr>
<tr>
<td>FSH µg/l</td>
<td>6–7</td>
<td>1.4 ± 0.13</td>
<td>1.36 ± 0.03</td>
<td>1.38 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>12–13</td>
<td>2.16 ± 0.20*</td>
<td>1.62 ± 0.33*</td>
<td>1.81 ± 0.42*</td>
</tr>
<tr>
<td>Oestradiol nmol/l</td>
<td>6–7</td>
<td>161.6 ± 105.6</td>
<td>1669.5 ± 1213.2</td>
<td>2578.0 ± 976.8</td>
</tr>
<tr>
<td></td>
<td>12–13</td>
<td>47.3 ± 2.2</td>
<td>1097.2 ± 623.3</td>
<td>2089.6 ± 851.4</td>
</tr>
</tbody>
</table>

*Significantly different (P < 0.05) from younger age within stage of the cycle.
PG = cloprostenol; FSH = follicle stimulating hormone.

luteal phase (P < 0.05) but with no change in oestradiol concentrations.

Experiment 2 (cohort)
Reduction in the concentration of progesterone after the injection of cloprostenol followed by an LH surge was observed in all animals, but the interval between cloprostenol-induced luteolysis to the onset of the LH surge (day = 0) was shorter in the young (43.2 ± 2.3 h, mean ± SD) cohort than in the older (58.0 ± 4.0 h) cohort (P < 0.05). The ovulation rate was similar (P > 0.05) between ages (2.0 ± 0 and 2.0 ± 0.4 for the young and old cohort, respectively), although it was more variable in the old cohort.

The pattern of FSH secretion (Figure 1) during the follicular phase was similar between the ages, but the older animals had higher baseline concentrations (P < 0.05) at the beginning of the follicular phase. The concentrations of FSH in both age groups peaked synchronously with the LH surge (between days 0 and 1) and started to increase from day 1 (P < 0.05). During the early luteal phase (days 2 to 6), the concentration of FSH was two- to threefold higher (P < 0.05) in the old cohort (Figure 1) and there was also greater variability between animals (Figure 2). The pattern of FSH secretion during the early luteal phase also differed between the ages. In the young cohort the concentration of FSH reached highest values of around 1.5 µg/l on day 2 and then began to decrease, while in the old cohort the mean FSH concentrations continued to rise to values above 2 µg/l on day 2 and remained high until day 6 (Figure 1).

The pattern of secretion of dimeric inhibin A was similar between ages (P > 0.05), but the overall mean concentrations of inhibin A tended to be higher in the young animals. In both age groups, the concentrations of inhibin A increased progressively during the follicular phase (P < 0.05), reaching maximum values at the onset of the LH surge, before declining markedly. During the luteal phase the concentrations of inhibin A started to increase earlier (P < 0.05) in the young cohort, reaching mean values over 0.5 µg/l on day 2, compared with day 3.5 in the old cohort. The concentrations of inhibin A were higher (P < 0.05) in the young animals during the luteal phase on day 15 (at the time of cloprostenol injection) and between days 1 and 2.5 of the new cycle (Figure 1).

The concentrations of oestradiol and androstenedione in ovarian venous blood (Figure 1) were not influenced by the age of the ewes (P > 0.05). They increased progressively after the luteal phase (P < 0.05) but with no change in oestradiol concentrations.
Figure 2. Dynamics of ovulatory follicles/CL (△△), and follicles from the first wave of follicular development (○●), ovarian secretion of oestradiol (●), androstenedione (●) and inhibin A (○) and concentration of follicle stimulating hormone (FSH) (○) in jugular venous blood in representative old (11 and 19) and young (68 and 72) animals during the follicular and early luteal stages of the oestrous cycle. Dotted line indicates time of the beginning of the luteinizing hormone surge.
injection of cloprostenol \( (P < 0.05) \) and reached maximum values at the time of LH surge \( (P < 0.05) \). By day 1, the concentration of these steroids had fallen to their lowest values observed during the cycle \( (P < 0.05) \) and subsequently started to increase on day 2 \( (P < 0.05) \). The levels of ovarian steroids remained stable from day 3 until day 5 before starting to decline \( (P < 0.05) \).

In both groups of ewes the mean concentration of progesterone in the peripheral blood was not influenced by age \( (P > 0.05) \) and remained low during the follicular phase and started to rise on day 4, progressively increasing until day 6 \( (P < 0.05) \), when it reached values around 5 nmol/l (Figure 1).

**Relationship between follicular development and hormonal secretion**

During the early luteal phase of the cycle, all ewes developed at least one large follicle. However, the number of follicles in this first wave was significantly higher \( (P < 0.05) \) in younger ewes \( (1.6 \pm 0.2 \text{, mean } \pm \text{SEM, } n = 5) \) than the older animals \( (1.0 \pm 0.0, n = 7) \). These large follicles emerged significantly later after the LH surge in the older animals \( (30.0 \pm 4.1 \text{ h and } 45.6 \pm 2.4 \text{ in the young and old cohort, respectively; } P < 0.05) \).

The relationship between follicular development during the first wave of follicles in the luteal phase and pattern of hormonal secretion is presented in detail in Figure 3, with the data being aligned to the time of emergence of the large follicles. The pattern of follicle growth was not influenced by the age of the animals \( (P > 0.05) \). In both age groups, the follicles grew progressively \( (P < 0.05) \) from the time of emergence until they achieved a diameter of around 5 mm, 2.5 days later, but they grew faster during the first day after emergence in the old cohort \( (P < 0.05) \).

The pattern of FSH secretion was significantly \( (P < 0.05) \) affected by the age of the animals. The concentrations of FSH in both age groups were high at the emergence of the large follicles and decreased as the follicles grew \( (P < 0.05) \) in the young cohort, while it remained high throughout in the old animals. The concentration of inhibin A was higher \( (P < 0.05) \) in the young cohort throughout the growing phase of the large follicles but remained similar \( (P > 0.05) \) once the follicles achieved a diameter around 5 mm.

The pattern of ovarian steroids was not influenced \( (P > 0.05) \) by age (Figure 3). The secretion of ovarian steroids increased as the large follicles grew, except that the concentration of androstenedione did not start to rise until 1 day after the emergence of the follicles in the older ewes \( (P < 0.05) \). There was a progressive decline \( (P < 0.05) \) in the secretion of oestradiol starting 3.5 days after the follicle emergence. The secretion of inhibin A also started to decrease at a similar time but the reduction was of a lesser magnitude. The first significant decline in large follicle diameter was not observed until a day later; therefore, the follicle persisted as a recognizable structure long after it had become atretic and had ceased to be a significant source of hormone secretion.

**Discussion**

Together the two experiments presented in this paper show that advanced age in sheep is associated with an increase in peripheral FSH concentrations, a decrease in ovarian inhibin A secretion and no change in ovarian steroid secretion. These hormonal changes are similar to those seen in peri-menopausal women (Sherman et al., 1976; Van Look, et al., 1977; Hansen et al., 1996; Klein et al., 1996) and are consistent with a scenario of depletion of the follicular population leading to a decline in inhibin secretion and hence an increase in FSH concentrations.

Increased concentrations of FSH at advanced age have been previously reported in rats (Meredith and Butcher, 1985) and women (Ebbiary et al., 1994). In women, this selective increase in FSH seems to be unrelated to the concentration of oestradiol in a similar fashion to the effects observed in this study, although in the human the increase in gonadotrophin secretion is associated with a decrease in the concentration of inhibin...
B (Klein et al., 1996) not inhibin A, as we observed in sheep. In women the pattern of dimeric inhibin secretion during the follicular phase of the menstrual cycle is well defined, with a predominance of the inhibin B form being associated with undifferentiated follicles at the beginning of the follicular phase and with a shift to the secretion of inhibin A as the follicles differentiate and the dominant follicle is established during the late follicular phase (Groome et al., 1996; Magoffin and Jakimiuk, 1997). In the rat the concentration of inhibin B is higher during metoestrous (recruitment) while inhibin A is predominant during pro-oestrous (follicle selection; Woodruff et al., 1996). The pattern of secretion of inhibin B in sheep is unknown and changes in inhibin B secretion could also contribute to the increase in FSH with age observed in this study. However, as granulosa cells from small undifferentiated follicles have been shown to secrete considerable quantities of inhibin A (Campbell et al., 1997), it is likely that species differences exist in the origin of the forms of inhibin secreted. In addition, in species that have continuous turnover of waves of follicular growth, such as sheep, inhibin A could be more important in regulating basal concentration of FSH, as inhibin A is 100-fold more potent than the B form in suppressing the release of FSH from ovine pituitary cells cultured in vitro (Robertson et al., 1996). Moreover, the same preparation of inhibin A assayed for biopotency in rat pituitary cells was only fivefold more efficient than inhibin B in suppressing FSH release in vitro, suggesting further species differences in FSH regulation (Robertson et al., 1996).

Serial measurements by high-resolution ultrasound revealed certain differences between the older and younger animals in the dynamics of follicle development. The fact that there were fewer small antral follicles available for development during the early luteal phase in the older ewes is consistent with an overall depletion in the number of follicles with age. However, during the follicular phase the number of ovulatory follicles and their maximum diameter was similar to that observed in young animals, although the number was more variable. These findings suggest that, although the pool of follicles is depleted in older animals, a greater proportion of antral follicles is promoted through to ovulation. Although there are no equivalent data in pre-menopausal women on the dynamics of follicle development, the increase in dizygotic twinning observed with age would suggest a similar mechanism (Tong and Short, 1998). It is tempting to speculate that the raised baseline levels of FSH are responsible for maintaining the development of nearly all available antral follicles as has been suggested in the mouse (Krarrup et al., 1969). Although the normal pattern of FSH secretion is maintained, the basal level is never suppressed below the threshold (about 1 µg/l) necessary to propel small antral follicles into the final growth phase of an ovulatory follicle. Thus, virtually every available follicle is promoted for ovulation and the selection process, which occurs in the normal follicular phase, is overridden. As a result, although the mean ovulation rate is maintained, the number of ovulatory follicles is more variable, which would explain the increased incidence of twinning in older women.

In sheep it is thought that inhibin regulates the basal concentration of FSH, while changes in oestradiol secretion are thought to be responsible for fluctuations during the cycle (Baird et al., 1991). The pattern of inhibin A secretion during the oestrous cycle is stable throughout the luteal phase, but increases above the baseline during the growth of large oestrogenic follicles during the follicular phase and early luteal phase (first wave). Inhibin A secretion is greatly reduced after the LH surge and this pattern of secretion is consistent with the lack of mRNA expression for both α and β subunits of inhibin A in large antral follicles during and after the LH surge (Engelhardt et al., 1993). The pattern of dimeric inhibin B secretion in sheep is less understood. Previous reports on the secretion of immunoreactive inhibin have shown that at least half of the inhibin secreted by the ovary originates from follicles other than the large oestrogenic follicle (dominant) (Campbell et al., 1991a; Mann et al., 1992). This observation is consistent with the pattern of expression of mRNA and protein for α, βA and βB subunits of inhibin which are located in the granulosa cells of the majority of non-ateitic antral follicles (Engelhardt et al., 1993, 1995; Braw-Tal, 1994).

In conclusion, in older sheep the elevated levels of FSH are associated with reduced ovarian secretion of inhibin A but with an unchanged level of oestradiol and ovulation rate compared with younger ewes. This suggests a relationship between the secretion of inhibin A and the total follicle population in the ovary, the reduction of which may result in an increase in the concentrations of FSH in a similar fashion to that observed in peri-menopausal women. Thus, the sheep could be a useful animal model to study the variations in the ovarian follicle population and hormone secretion in order to obtain hormonal predictors of the onset of menopause and its pathological expression, premature ovarian failure.

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