Direct effects of ovine follicular fluid on ovarian steroid secretion and expression of markers of cellular differentiation in sheep

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The objective of this study was to assess the effect of ovine follicular fluid (FF) treatment (with or without FSH replacement) during the late follicular phase on plasma concentrations of gonadotrophins and the development of the ovulatory follicle. Ovarian steroid secretion and expression of mRNA encoding inhibin α and β_A , β_B subunits, P450 aromatase and P450 17 α -hydroxylase were used as endpoints. After induction of luteolysis by injection of 100 μ g cloprostenol on days 10–12, Scottish Blackface ewes were allocated to one of three groups: (1) control ($n = 7$): no further treatment; (2) FF ($n = 9$): subcutaneous injections of 3 ml steroid-free ovine follicular fluid at 9 h intervals, 18 and 27 h after cloprostenol injection; (3) FF + FSH ($n = 8$): injections of follicular fluid as above plus subcutaneous injections of 0.36 iu ovine FSH at 6 h intervals, 18, 24, and 30 h after cloprostenol injection. Jugular venous blood samples were obtained via indwelling cannulae at 6 h intervals from 0 to 36 h after cloprostenol injection, and at 10 min intervals from 12 to 18 h (control phase) and from 30 to 36 h after cloprostenol injection (treatment phase). At laparotomy, 36 h after cloprostenol injection, ovarian venous blood was collected and ovaries were removed and processed for *in situ* hybridization. Plasma concentrations of FSH, luteinizing hormone (LH) and oestradiol were determined by radioimmunoassay. Follicular fluid treatment resulted in a decrease ($P < 0.001$) in FSH concentrations associated with an acute decrease in ovarian steroid secretion ($P < 0.01$) and a specific depression in P450 aromatase, ($P < 0.001$), inhibin–activin β_B subunit ($P < 0.05$) and thecal LH receptor ($P < 0.001$) expression. Follicular fluid treatment had no effect on inhibin–activin α and β_A subunit or P450 17 α -hydroxylase expression. FSH co-treatment with follicular fluid restored circulating FSH concentrations to normal values and reversed some of the effects of follicular fluid (androstenedione, testosterone and progesterone secretion, and inhibin β_B and thecal LH receptor expression) but not oestradiol secretion or P450 aromatase expression. It was concluded that the actions of follicular fluid are mediated via both central effects on pituitary FSH secretion and by direct ovarian effects on granulosa cell aromatase activity. The results indicate that follicular fluid contains a factor that inhibits aromatase activity of granulosa cells directly and may play a role in the selection of the dominant follicle.

Introduction

In sheep, the number of ovulatory follicles and, hence, the ovulation rate, is rigorously controlled, with most breeds having a characteristic ovulation rate between one and three

(Scaramuzzi *et al.*, 1993). The use of real-time ultrasonography to study the pattern of follicle development in sheep throughout the oestrous cycle has revealed that large ovulatory sized, or dominant, follicle development occurs throughout the luteal phase as a series of 4–6 day periodic waves, with the number of follicles per wave being similar to the ovulation rate of that breed (Ginther *et al.*, 1995; Souza *et al.*, 1997a). Furthermore, these studies have shown that ovulatory follicles can be either derived from the large follicles present in the ovary at the time of luteal regression or recruited from the small antral follicle pool during the first 12–24 h of the follicular phase depending on the stage of the follicular wave at the time of luteolysis (Souza *et al.*, 1997b).

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Fig. 1. Schematic representation of experimental design and sampling frequency. PG: injection of 100 µg cloprostenol; FF: subcutaneous injections of 3 ml steroid-free ovine follicular fluid; FSH: subcutaneous injections of 0.36 IU ovine FSH; OVX: ovariectomy.

However, understanding of the mechanisms that control these cycles of follicular recruitment, selection and dominance are incomplete.

It has been hypothesized that fluctuations in peripheral FSH concentrations, in response to variations in the degree of negative feedback provided by oestradiol and inhibin secretion, represent the mechanism of follicular recruitment and dominance (Baird, 1983) and much data have been accumulated to support this hypothesis. In both cattle (Adams *et al.*, 1992; Gong *et al.*, 1993) and sheep (Ginther *et al.*, 1995; Souza *et al.*, 1997a), there is compelling evidence that the emergence of a new follicular wave is associated with a transient increase in FSH, and that periods of follicular dominance are associated with basal FSH concentrations. Furthermore, studies using experimental models in which GnRH analogues have been used to suppress endogenous gonadotrophins with subsequent hormone replacement have shown that FSH alone can stimulate the recruitment and development of ovulatory sized follicles (Picton *et al.*, 1990; Gong *et al.*, 1996; Campbell *et al.*, 1998), that dominant follicles are critically dependent on FSH support (Campbell *et al.*, 1999) and that dominant follicles can transfer their gonadotrophic requirement from FSH to LH if it is delivered in low amplitude, high frequency pulses (Campbell *et al.*, 1999).

While evidence for the FSH-threshold hypothesis of follicular dominance is compelling, an alternative hypothesis proposes that a dominant follicle actively secretes a factor or factors that directly suppress the development of subordinate follicles. Treating ewes with steroid-free follicular fluid late in the follicular phase, for more than 24 h after the induction of luteolysis, results in a delay in the onset of oestrus (McNeilly, 1984, 1985; Henderson *et al.*, 1986, 1988). Further studies into this phenomenon in ewes with an ovarian autotransplant showed that follicular fluid treatment was associated with a rapid decrease in ovarian hormone secretion (Baird *et al.*, 1990; Campbell *et al.*, 1991a) that results from acute induction of atresia of the ovulatory follicles (Campbell *et al.*, 1991a). However, as steroid-free follicular fluid treatment is associated with a decrease in FSH (McNeilly, 1984, 1985; Wallace *et al.*, 1985; Wallace and McNeilly, 1985; Baird *et al.*, 1990), owing to its inhibin content

(Leversha *et al.*, 1987), these inhibitory effects of follicular fluid can be satisfactorily explained by the FSH-threshold hypothesis.

More compelling evidence for the dominant factor hypothesis comes from the demonstration that inhibin-free follicular fluid can delay oestrus (Law *et al.*, 1992; Baxter *et al.*, 1995), suppress ovarian steroid secretion (Campbell *et al.*, 1991a) and induce follicular atresia (Campbell *et al.*, 1991a; Law *et al.*, 1992) without inducing significant changes in peripheral FSH concentrations. Furthermore, it has been demonstrated that direct ovarian infusion of follicular fluid can suppress ovarian function without any change in FSH (Campbell *et al.*, 1996a) and that fractions of follicular fluid can inhibit FSH-dependent differentiation of granulosa cells *in vitro* (Baxter *et al.*, 1995). The aim of the present study was to investigate this activity by assessing the effect of follicular fluid treatment, with or without FSH replacement, on ovarian steroid secretion and expression of mRNA encoding cytochrome P450 aromatase (P450_{arom}), cytochrome P450 17 α -hydroxylase (P450_{17 α}), inhibin-activin α , β_A and β_B subunits and LH receptor, which were used as key markers of follicular differentiation.

Materials and Methods

Animals

Experiments were conducted in accordance with the Animal (Scientific Procedures) Act of 1986 (UK). During the breeding season (October–January), the oestrous cycles of 24 mature Scottish Blackface ewes (ovulation rate 1.3 ± 0.1) were synchronized by insertion of intravaginal progestagen sponges (Dunlop, Dumfries) for 12–14 days followed by injection of 100 µg prostaglandin F_{2 α} analogue, cloprostenol (Estrumate, Dunlop) 10 days after sponge removal.

Experimental design

A summary of the experimental design is provided (Fig. 1). All animals received a second injection of cloprostenol

Table 1. Details of ³⁵S-labelled riboprobe templates used for *in situ* hybridization

Probe	Vector	Size (bp)	Endonuclease		Polymerase		Reference
			Sense	Antisense	Sense	Antisense	
hInhibin α	pGEM3Zf(+)	360	EcoR I	Hind III	SP6	T7	Engelhardt <i>et al.</i> 1993
hInhibin β_A	pBluescript II SK+	618	BSSH II	BSSH II	T7	T3	Engelhardt <i>et al.</i> 1993
hInhibin β_B	pGEM3Zf(+)	623	EcoR I	BamH I	SP6	T7	Harkness and Baird, 1997
oLH _R	pBluescript II sk +/-	730	Xba I	EcoR V	T7	T3	Xu <i>et al.</i> , 1995a
bP450 _{17α}	pBluescript II sk +/-	400	EcoR V	Xba I	T3	T7	Xu <i>et al.</i> , 1995b
oP450 _{arom}	pGEM T	429	Apa I	Not I	SP6	T7	See text

to induce the follicular phase for the experimental cycle, 11 days after the first (time = 0 h). Jugular venous blood samples were obtained via indwelling cannulae at 6 h intervals from 0 to 36 h after cloprostenol injection, and at 10 min intervals 12–18 h and 30–36 h after cloprostenol injection. Treatments were assigned as follows: (1) control ($n = 7$): no further treatment; (2) FF ($n = 9$): subcutaneous injections of 3 ml steroid-free ovine follicular fluid at 9 h intervals, 18 and 27 h after cloprostenol injection; (3) FF + FSH ($n = 8$): injections of follicular fluid as above plus subcutaneous injections of 0.36 iu ovine FSH at 6 h intervals, 18, 24, and 30 h after cloprostenol injection. The amounts and timing of FSH were designed to restore the concentrations of FSH to those found in control ewes.

At 36 h after the second injection of cloprostenol, timed samples of ovarian venous blood were collected and ovaries were removed as described by Campbell *et al.* (1991b) and Engelhardt *et al.* (1993). The time chosen to collect ovaries was based on previous studies of the time course of hormone changes after injection of follicular fluid and corresponded to the nadir in secretion of oestradiol and androstenedione before ovulation or collapse of the follicle (Baird *et al.*, 1990; Campbell *et al.*, 1991a). Briefly, midventral laparotomies were performed under general anaesthesia, reproductive tracts were exteriorized and ovarian veins were cannulated. A timed 10 ml sample of ovarian blood was collected from the left and right ovarian veins by gentle suction with a syringe. Both ovaries of each animal were then removed, the presence of large follicles (> 4 mm) noted and ovaries were fixed overnight in freshly prepared 4% (v/v) paraformaldehyde in PBS, pH 7.4 (PFA).

After fixation, ovaries were cut longitudinally in two or four slices, care being taken to cut through the largest follicles on each ovary. The tissue was then dehydrated by passage through a graded series of alcohols and embedded in paraffin wax. Serial sections (5 mm thickness) were mounted on slides coated with 3-aminopropyltriethoxysilane (TESPA; Sigma, St. Louis, MO), incubated overnight at 50°C, and then processed for *in situ* hybridization as described by Harkness and Baird (1997).

Preparation of follicular fluid and FSH

Collection and preparation of the ovine follicular fluid used in these experiments have been described by Baird *et al.*

(1990). Briefly, follicular fluid collected from abattoir-derived ovaries was charcoal-stripped and passed through a C18 Sep-Pak cartridge to remove steroids, filter-sterilized and frozen until use. The follicular fluid contained 3235 μ g inhibin ml⁻¹ (p1-26a) and had a bioactivity of 8076 U ml⁻¹ (Tsonis *et al.*, 1986). The treatment regimen (3 ml follicular fluid at 9 h intervals) was intended to reduce FSH to approximately 50% of control concentrations.

The experiment was performed in two trials, 2 months apart. In the first trial ($n = 15$), the FSH used was NIAMDD-oFSH-14 (biopotency = $9 \times$ NIH-FSH-S1) whereas, for the second trial ($n = 9$), USDA-oFSH-19-SIAFP (biopotency = $94 \times$ NIH-FSH-S1) was used. For both trials, the appropriate amount of FSH was dissolved in sterile 0.9% saline containing 1% normal sheep serum before injection. The amount of FSH infused was chosen to maintain the FSH concentration at the amount found before the injection of prostaglandin, that is, during the luteal phase of the cycle.

Radioimmunoassays

Plasma concentrations of FSH, LH, and oestradiol were determined by previously established radioimmunoassays (McNeilly and Fraser, 1987; McNeilly *et al.*, 1989; Campbell *et al.*, 1990). The sensitivities of the assays for FSH, LH and oestradiol were 0.3 μ g l⁻¹ (USDA-oFSH-SIAFP-RP2), 0.2 μ g l⁻¹ (NIH-LH-S18) and 120 pg l⁻¹ respectively. The intra- and interassay coefficients of variation of the assays used were < 15% in the ED20–80 range.

The total rate of ovarian steroid secretion was calculated by multiplying oestradiol concentrations in ovarian venous plasma by ovarian plasma flow rate (ovarian blood flow per minute corrected for haematocrit) for each ovary and summing these values for the left and right ovaries (Collett *et al.*, 1973).

Template generation and cRNA probe synthesis

Details of the cDNA templates and cRNA probe synthesis are presented (Table 1). With the exception of the P450_{arom} cDNA templates, the preparation of these templates has been described elsewhere (see Table 1). For P450_{arom}, a 429 base pair (bp) cDNA encoding ovine P450_{arom} was generated by reverse transcriptase–polymerase chain reaction using ovine

preovulatory follicle RNA and primers corresponding to highly conserved sequences present in the nucleotide sequence of bovine P450_{arom} (Genbank accession number U18447). The ovine P450_{arom} cDNA (corresponding to nucleotides 1406–1834 of the bovine P450_{arom}) was subsequently ligated into the pGEM T vector (Promega UK Ltd, Southampton) and sequenced to verify identity. The ovine P450_{arom} cDNA was 95% identical to the reported sequence of bovine P450_{arom}. ³⁵S-labelled sense and antisense riboprobes were generated from their cDNA templates using methods described by Harkness and Baird (1997).

In situ hybridization

All *in situ* hybridization procedures were performed as described by Harkness and Baird (1997). Sections were deparaffinized twice in HistoClear (National Diagnostics, Atlanta, GA), rehydrated through a graded series of ethanols and post-fixed in 4% (v/v) PFA. The sections were washed and digested with proteinase K, (20 µg ml⁻¹; Sigma) in a 100 mmol Tris l⁻¹–10 mmol EDTA l⁻¹ buffer. Sections were washed and refixed in 4% (v/v) PFA, washed twice in 0.25% acetic anhydride in 0.1 mol triethanolamine hydrochloride l⁻¹ and washed again in PBS and saline, dehydrated through a graded series of ethanols (50, 70, 85, 95 and 100%) and air dried.

The labelled probes were diluted to a concentration of 5×10^5 c.p.m. per slide, with a hybridization buffer (62.5% deionised formamide; 25% of a 50% dextran sulphate solution; 7.5% 5 mol NaCl l⁻¹; 1% 1 mol Tris l⁻¹, pH 8.0; 0.2% 500 mmol EDTA l⁻¹; 2.5% 50× Denhardt's solution; 1% 1 mol dithiothreitol l⁻¹ and 0.3% ultrapure water) heated to a temperature of 80°C for 2 min and cooled on ice before application to sections at 10 ml per slide. Sections were overlaid with coverslips made from Gel Bond (FMC Bioproducts, Rockland, ME) and the slides were incubated overnight in a humidified chamber saturated with 2.5× standard sodium citrate and 50% formamide at 55°C. After hybridization, the sections were washed in 5× SSC with 0.01 mol dithiothreitol l⁻¹ at 55°C, in 2× SSC with 50% deionized formamide and 0.1 mol dithiothreitol l⁻¹ at 65°C and then three times in NTE buffer (0.5 mol NaCl l⁻¹, 10 mmol Tris l⁻¹, 5 mmol EDTA l⁻¹, pH 7.5) at 37°C.

Sections were then treated with ribonuclease type III A (Sigma, 40 mg ml⁻¹ in NTE) at 37°C, then re-washed in NTE. The wash in 2× SSC with 50% formamide and 0.1 mol dithiothreitol l⁻¹ at 65°C was repeated. Sections were then washed in buffers containing decreasing amounts of salt solutions at room temperature (2× and 0.1× SSC) and then dehydrated through a graded series of ethanols (50 and 85% containing 0.3 mol NH₄ acetate l⁻¹; 100% twice) and left to air dry.

Autoradiographic dipping in liquid emulsion was performed in a dark room with a safety light. Sections were dipped in Kodak NBT-2 (Rochester NY) and exposed for 1–5 weeks at 4°C, developed in Kodak D-19 developer at 15°C, and fixed in Kodafix at room temperature. Slides were counterstained with haematoxylin (BDH, Glasgow) dehydrated and mounted in Pertex mounting media (CellPath, Hemel Hempstead).

Slides were examined under dark field using an Olympus microscope attached to a video camera and computer. The concentration of silver grains was measured using two different programmes. The silver grains of the riboprobes of inhibin α , β_A and β_B , aromatase and LH receptor were measured at 10⁶ cm⁻³, using an image analysis programme, Olympus PC-Task Manager Version 1.20A, Cue 2 Image Analysis Morphometry. The measurements of the silver grain density for the 17 α -hydroxylase were generated using Image-Pro Plus (Microsoft Windows 95) at 10⁶ cm⁻². These results were then converted to 10⁶ cm⁻³.

Five fields per section were analysed with a coefficient of variation of < 15%. One section per follicle was analysed for each probe and all sections were included in a single run for each probe. Sense probes were used in parallel with antisense probes on alternate sections to control for nonspecific hybridization. No signal was observed with sense probes in any tissue (data not shown). Sheep ovaries from untreated adult sheep during the luteal phase were included in each run as positive controls.

Statistical analysis

Because ovaries were not serially sectioned, follicles in a section were considered a sample of those in that particular ovary. Follicle diameters were measured to the nearest 0.5 mm using an eyepiece graticule, and then grouped into four size categories: 1–2 mm, 2–3 mm, 3–4 mm and > 4 mm. In the case of the larger follicles, the diameter was measured in the section in which it was largest. Treatment effects on follicle number were tested using one way analysis of variance after $\sqrt{(x + 0.5)}$ transformation.

Comparison of plasma gonadotrophin concentrations during 10 min sampling periods, steroid secretion rates and values for mRNA expression were made using one way analysis of variance on log-transformed data, whereas repeated measures analysis of variance was used to assess changes in gonadotrophin concentrations over time (6 h samples). When initial *F* tests were significant (*P* < 0.05), Duncan's multiple range procedure was used to compare individual means. LH pulse characteristics (frequency and amplitude) were determined using the Munro pulse analysis programme (Elsevier, Biosoft, Haddington, East Lothian) and compared using one way analysis of variance as described above. Hormone concentrations are presented as untransformed means $\pm$ SEM, whereas mRNA expression is presented as log grains cm⁻³.

Results

FSH and LH concentrations in peripheral blood

Before treatment with follicular fluid, there were no inter-group differences in plasma FSH concentrations in the 6 h samples (data not shown) or in samples collected over the initial period of intensive blood sampling, from 12–18 h after cloprostenol injection (Fig. 2). Follicular fluid treatment caused a 36% reduction in plasma FSH concentrations

Fig. 2. Mean $\pm$ SEM concentrations of (a) FSH and (b) LH before (■; 12–18 h after cloprostenol injection; control phase) and after (□; 30–36 h after cloprostenol injection; treatment phase) treatment. *** $P < 0.001$ for comparison between control and treatment phases within a treatment group. FF: follicular fluid.

($P < 0.0001$), which was reversed by concomitant treatment with FSH (Fig. 2).

Mean LH concentrations over the initial period of intensive blood sampling were lower ($P < 0.001$) in the FF group, before follicular fluid treatment. However, neither follicular fluid nor follicular fluid plus FSH treatment had any significant effect on mean LH concentrations (Fig. 2), interpulse interval (1.9 ± 0.7 , 2.3 ± 0.6 , 3.8 ± 0.9 h for control, FF and FF + FSH groups, respectively) or pulse amplitude (1.7 ± 0.3 , 0.9 ± 0.2 , 1.2 ± 0.5 μ g l⁻¹ for control, FF and FF + FSH groups, respectively) over the treatment period, 30–36 h after cloprostenol injection.

Size distribution of follicles

The size distribution of follicles exhibited the expected pattern (Turnbull *et al.*, 1977) with a rapid decrease in the number of follicles as follicle size increased (data not shown). There were no differences in the number of follicles among the three treatment groups, except that there were more large (> 4 mm) follicles in the FF + FSH group (2.13 ± 0.30) than in the control or FF groups (1.29 ± 0.18 and 1.33 ± 0.17 , respectively; $P < 0.05$).

Ovarian effects: secretion of ovarian steroids

As reported by Campbell *et al.* (1991a) and Mann *et al.* (1992), ovarian steroid secretion from ovaries containing no large follicles was minimal and, as a result, only steroid secretion from ovaries containing at least one large (> 4 mm) follicle has been presented (Fig. 3). When compared with untreated controls, treatment with follicular fluid resulted in a decrease ($P < 0.05$) in ovarian oestradiol, androstenedione, testosterone and progesterone secretion. Combined treatment with follicular fluid and FSH was largely able to prevent this depressive effect of follicular fluid on ovarian androstenedione, testosterone and progesterone secretion, but oestradiol secretion remained below ($P < 0.05$) control values.

Ovarian effects: mRNA expression for differentiative markers

Expression of mRNA encoding P450_{arom} was only detected in the granulosa cells of large follicles (Fig. 4). Treatment with follicular fluid caused a marked decrease ($P < 0.001$) in P450_{arom} expression and combined treatment with follicular fluid and FSH was unable to reverse this effect (Fig. 4). In contrast, P450_{17 α} was expressed in the thecal cells of all follicles (Fig. 4), although the expression was greater ($P < 0.01$) in large than in small follicles (4.8 ± 0.6 versus 2.8 ± 0.3 log grains cm⁻³, respectively). Neither follicular fluid nor follicular fluid plus FSH treatment had any effect on the expression of P450_{17 α} in large (Fig. 4) or small follicles (data not shown).

Inhibin-activin α , β_A and β_B subunits were expressed exclusively in the granulosa cells (Fig. 5). Expression of inhibin α was greater in large than in small follicles (5.5 ± 0.7 versus 3.4 ± 0.3 log grains cm⁻³; $P < 0.01$) but was unaffected by follicular fluid and follicular fluid plus FSH treatment (Fig. 4). Similarly, expression of inhibin β_A was greater in large than in small follicles (5.4 ± 0.5 versus 0.6 ± 0.2 log grains cm⁻³; $P < 0.001$) and was unaffected by follicular fluid and follicular fluid plus FSH treatment (Fig. 4). Inhibin-activin β_B subunit expression was also confined mainly to large follicles (large versus small: 1.3 ± 0.4 versus 0.3 ± 0.2 log grains cm⁻³; $P < 0.05$) but was decreased ($P < 0.05$) by treatment with follicular fluid, and this effect was reversed by treatment with follicular fluid plus FSH (Fig. 5).

LH receptor was expressed in the thecal cells of all follicles, with follicular size having no effect on the amount of expression (6.2 ± 1.0 versus 4.4 ± 0.5 log grains cm⁻³ in large and small follicles from controls, respectively). In both large (Fig. 5; $P < 0.001$) and small (1.8 ± 0.4 log grains cm⁻³; $P < 0.01$) follicles, treatment with follicular fluid decreased thecal LH receptor expression, and this effect was largely prevented by treatment with follicular fluid plus FSH in both large (Fig. 4; $P = 0.3$) and small follicles (2.5 ± 0.5 log grains cm⁻³; $P = 0.09$) when compared with controls. LH receptor was only intensely expressed in the granulosa cells in a small number of large antral follicles, and so these data were not analysed for treatment effects.

Fig. 3. Ovarian secretion rates of (a) oestradiol, (b) progesterone, (c) androstenedione and (d) testosterone determined before ovariectomy in control ewes (C; □; $n = 7$), ewes treated with follicular fluid (FF; ■; $n = 9$) and ewes treated with follicular fluid plus FSH (FF + F; ▨; $n = 8$). Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ for comparison with control.

The follicular fluid-induced decrease in mRNA expression was not associated with morphological evidence of atresia (sloughing or thinning of granulosa layer and pyknotic nuclei), and none of the large follicles in the sheep treated with follicular fluid appeared to be atretic.

Discussion

The results of the present study confirm previous reports that treatment of ewes with steroid-free ovine follicular fluid during the follicular phase results in an acute decrease in ovarian steroid secretion that is associated with a 36% decrease in peripheral FSH concentrations (Baird *et al.*, 1990; Campbell *et al.*, 1991a). However, in addition, the present study shows that this decrease in steroid secretion is associated with a specific decrease in P450_{arom}, inhibin-activin β_b subunit and thecal LH receptor expression. Furthermore, the fact that FSH co-treatment restored circulating FSH concentrations to normal values and reversed some of the effects of follicular fluid (androstenedione, testosterone and progesterone secretion and inhibin β_b and thecal LH receptor expression) but not others (oestradiol secretion and P450_{arom} expression) indicates that the actions of follicular fluid are mediated via both central effects on pituitary FSH secretion and by direct ovarian effects on granulosa cell aromatase activity.

The possibility that follicular fluid exerts its actions via both direct and indirect mechanisms helps explain the wide

range of effects observed after treatment with follicular fluid. In addition to ovarian steroid secretion, treatment with follicular fluid during the follicular phase reduces the number of granulosa cells, oestradiol content, aromatase activity and *in vitro* gonadotrophin responsiveness in large follicles (Henderson *et al.*, 1986). Furthermore, by using animals with ovarian autotransplants, treatment with ovine follicular fluid has been shown to acutely inhibit ovarian immunoreactive inhibin secretion (Baird *et al.*, 1990; Campbell *et al.*, 1991a). One of the major strengths of the design used in the present study is its ability to differentiate between direct and FSH-dependent actions of follicular fluid and to identify the mechanism of action at a molecular level.

For instance, one of the most puzzling aspects of our previous studies was the ability of follicular fluid to suppress ovarian androgen secretion acutely (Baird *et al.*, 1990; Campbell *et al.*, 1991a), as androgens are produced solely by thecal cells that are under the trophic control of LH. The fact that P450_{17 α} expression was unaffected by follicular fluid, despite a marked decrease in both androstenedione and testosterone secretion, indicates that follicular fluid is not acting through modulation of the activity of this enzyme. Rather, the fact that thecal LH receptor expression was decreased by follicular fluid, allied with the knowledge that ovarian androgen secretion is acutely responsive to LH (Baird *et al.*, 1981), indicates that follicular fluid affects androgen secretion through modulation of LH receptor expression, a hypothesis that is in agreement with the

Fig. 4. Expression of RNA for (a) P450 aromatase (b) inhibin α subunit, (c) inhibin β_A subunit, (d) inhibin β_B subunit, (e) P450 17 α -hydroxylase and (f) thecal LH receptor in the granulosa (a-d) and theca (e-f) cell layers of large (> 4 mm in diameter) ovarian follicles in control ewes (C; $\square$; $n = 7$), ewes treated with follicular fluid (FF; $\blacksquare$; $n = 5$) and ewes treated with follicular fluid plus FSH (FF + F; $\square$; $n = 6$). Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for comparison with control.

observation that FSH co-treatment leads to a reversal of the effects of follicular fluid on both LH receptor expression and ovarian androgen secretion. Similarly, as follicular progesterone secretion is also under the acute trophic control of LH (Baird *et al.*, 1976) it is likely that follicular fluid inhibits progesterone secretion via the same mechanism. However, the fact that FSH treatment is able to affect LH

receptor expression on thecal cells, which are devoid of FSH receptors (Carson *et al.*, 1979; McNatty *et al.*, 1986; Eckery *et al.*, 1996) supports the notion that granulosa cell-derived paracrine factors can modulate thecal function (Smyth *et al.*, 1993).

Owing to the interference of the inhibin contained within the follicular fluid that was injected, in this particular study

Fig. 5. *In situ* hybridization cDNA probes of sheep follicles in follicular phase: (a–f) control; (g–l) after treatment with ovine follicular fluid (oFF); (m–r) after treatment with oFF + FSH. (a,g,m) Haematoxylin and eosin staining; hybridization with cDNA for (b,h,n) aromatase, (c,i,o) β_1 inhibin, (d,j,p) β_2 inhibin, (e,k,q) LH receptor, and (f,l,r) P450 17 α -hydroxylase. The distortion of anatomy is an artefact of processing. Scale bar represents 0.5 mm.

there has been no attempt to determine the effect of follicular fluid treatment on ovarian inhibin secretion. However, by taking repeated samples of ovarian venous blood from ewes with an ovarian autotransplant, it has been shown that ovarian secretion of total immunoreactive inhibin is acutely suppressed by follicular fluid (Baird *et al.*, 1990; Campbell *et al.*, 1991a). As treatment with follicular fluid in the present experiment had no effect on inhibin α or β_A , but depressed β_B expression, it appears likely that this decrease in immunoreactive inhibin is due to a decrease in inhibin B rather than inhibin A secretion. This is a surprising finding, since inhibin A appears to be the product of large antral follicles in a number of species, including rats (Woodruff *et al.*, 1996) and humans (Groome *et al.*, 1996; Souza *et al.*, 1997c), and inhibin A and aromatase are often co-expressed in sheep (B. K. Campbell and D. T. Baird, unpublished). However, little is known about the pattern of secretion of inhibin B in sheep and determination of its relative importance must await the development of specific assays. Nevertheless, the ability of FSH to reverse the depressive effect of follicular fluid on follicular β_B expression is consistent with a number of studies *in vivo* (Tsonis *et al.*, 1986; McNeilly *et al.*, 1989; Campbell *et al.*, 1998) and *in vitro* (Campbell *et al.*, 1996b) showing that inhibin secretion in sheep is FSH responsive.

The ability of a follicle to secrete oestradiol is one of the key determinants of follicular health (Baird and McNeilly, 1981) and the level of P450_{arom} expression is a key marker of granulosa cell differentiation. Oestradiol production by granulosa cells, both *in vivo* (McNatty *et al.*, 1985; Campbell *et al.*, 1998) and *in vitro* (Campbell *et al.*, 1996a), is stimulated by FSH, whereas withdrawal of FSH support *in vivo*, in the absence of LH, leads to an acute decrease in oestradiol secretion and atresia of dominant follicles (Campbell *et al.*, 1999). In the present experiment, the inability of FSH to reverse the depressive effects of follicular fluid on both oestradiol secretion and P450_{arom} expression indicate that follicular fluid contains a factor, or factors, that act directly on granulosa cells to inhibit aromatase (Campbell *et al.*, 1991a, 1996a). The factors mediating this effect, and their mechanism of action are still unknown but potential candidates include follicle regulatory protein (diZerega *et al.*, 1982; Ono *et al.*, 1986), epidermal growth factor (Murray *et al.*, 1992), transforming growth factor α (Campbell *et al.*, 1994), insulin-like growth factor-binding protein (Shimasaki *et al.*, 1990), inhibin (Campbell *et al.*, 1996a) and granulosa cell inhibitory factor (Hynes *et al.*, 1996). Whether these factors play a role in the selection of the dominant follicle requires further experimentation.

While the results of the present experiment show clearly that FSH was not able to reverse fully the effects of follicular fluid, previous studies from our laboratory have shown that larger doses of FSH can prevent the follicular fluid-induced delay in the onset of oestrus in normal ewes (McNeilly 1985), and that follicular fluid is unable to prevent FSH stimulating follicle development in GnRH-agonist-suppressed ewes (McNeilly *et al.*, 1991). Thus, since the action of the inhibitor can be overcome by high doses of FSH, these findings indicate that the inhibitor ultimately modulates an FSH-dependent process. In the present and in previous studies (Henderson *et al.*, 1986), treatment with follicular fluid had

no effect on the number or size distribution of follicles at 24–48 h after cloprostenol injection. However, using a treatment regimen similar to that of the present study, Campbell *et al.* (1991a) reported that the number of large follicles (as determined by daily ultrasound scanning) decreased markedly by 72 h after cloprostenol injection in ewes treated with follicular fluid. Together, these results indicate that treatment with follicular fluid eventually induces atresia in the preovulatory follicle, that functional changes are detectable within 24 h of induction of luteal regression, and that actual physical regression of the follicle is evident by 72 h after cloprostenol injection.

In summary, the decrease in FSH concentrations after treatment with steroid-free ovine follicular fluid during the follicular phase results in an acute decrease in ovarian steroid secretion, which is associated with a specific depression in P450_{arom}, inhibin-activin β_B subunit and thecal LH receptor expression. FSH co-treatment was able to restore circulating FSH concentrations to normal values and reverse some of the effects of follicular fluid (androstenedione, testosterone and progesterone secretion and inhibin β_B and thecal LH receptor expression) but not others (oestradiol secretion and P450_{arom} expression). It is concluded that the actions of follicular fluid are mediated via both central effects on pituitary FSH secretion and by direct ovarian effects on granulosa cell aromatase activity. Further investigations into the identity of this inhibitory activity and its mechanism of action may shed light on the endocrine and paracrine conditions required for the prevention of atresia of follicles at this advanced stage of development.

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